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Membrane Bound IL21 Promotes Natural Killer Cell Expansion Through mIR 124-3p Mediated Regulation

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MEMBRANE BOUND IL21 PROMOTES NATURAL KILLER CELL EXPANSION THROUGH MIR 124-3P MEDIATED REGULATION

A

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Dedication

To the cancer survivors and their families in the hope that there would be cure,

One day soon…

Acknowledgements

I would like to start my acknowledgements with the sincerest gratitude to my mentors, colleagues, friends and most importantly family for their unwavering support, encouragement, patience and guidance throughout the course of my graduate education.

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Finally, my gratitude to the God Almighty for providing me with constant guidance, hope and opportunities and leading me through this path.

Membrane Bound IL21 Promotes Natural Killer Cell Expansion Through miR 124-3p Mediated Regulation

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Advisory Professors: Eugenie Kleinerman, M.D. & Dean A. Lee, M.D., Ph.D.

Natural Killer (NK) cells are cells of the innate immune system that act as first line of defense against viral infections and participate in tumor immune surveillance. NK cells do not cause graft versus host disease (GvHD), or require prior antigen exposure to exert anti-tumor activity, hence are an attractive choice for immunotherapy applications. NK cells comprise a mere 1-32% (6% median) of peripheral blood cells, hence ex vivo expansion of NK cells is critical for adoptive immunotherapy; various expansion methods for NK cells have been explored over the decades. We developed a robust platform for expansion of human NK cells using K562 expressing membrane bound IL-21 (K562.mbIL21), and showed that K562.mbIL21 enables significantly higher expansion of functionally potent NK cells compared to K562.mbIL15. Both membrane bound IL15 (mbIL15) and membrane bound IL21 (mbIL21) expanded NK cells are currently in several Phase I/II clinical trials. K562.mbIL21 surpasses all other NK cell expansion methods in its rate of NK cell expansion, as well as cytokine secretion, cytotoxicity, telomere length and MTOC synapse formation, yielding highly activated non-senescent cells.

IL-15 and IL-21 signaling are essential for proliferation and activation of NK cells in vivo, yet mbIL21 far exceeds that of mbIL15 based expansion ex vivo. This could be due to promotion of telomerase activity and telomere length by Stat 3 due to IL-21 signaling,

however this may not be the only determining factor. The observed differences between these 2 expansion systems could be caused by miRNAs, crucial regulators of post-transcriptional gene regulation. The role of miRNA in NK cell biology is an emerging area of interest and their effect on regulation of protein expression and function during expansion of NK cells remains unexplored. We hypothesize that *activating and expanding human NK cells on membrane bound IL15 and membrane bound IL21 will lead to differential gene expression mediated by miRNAs and subsequent observed differential expansion potential in these cells.* Thus, my project's goal is to identify miRNA that regulate the superior proliferation and function of NK cells produced by IL-21 signaling as well as to delineate the pathways that enable IL-21 signaling in achieving this effect.

To accomplish this, we initiated an exploratory study and evaluated the global changes in differential gene, protein and miRNA between mbIL15 and mbIL21 expanded NK cells and identified BCL2L11, Bim and miR 124-3p to be the most differentially expressed factors, respectively ($p < 0.01$), with highest expression in mbIL15 NK cells. This indicated that miR 124-3p and Bim downregulation might mediate a proliferative and anti-apoptotic advantage in mbIL21 NK cells, and consequently better expansion. MiR 124-3p is recently shown to be a tumor suppressor miRNA, with high expression in normal cells; downregulation of miR 124-3p induces tumorigenesis, proliferation and suppression of apoptosis. We further correlated and validated the findings by knockdown of miR124-3p in NK cells, and showed its effect on *BCL2L11*, Bim and Stat3 expression. We discovered that knockdown of miR124-3p decreased *BCL2L11* and Bim expression, and increased telomerase reverse transcriptase (TERT), Stat 3 and Stat 3 phosphorylation and consequent increase in proliferation, decreased apoptosis and increased overall expansion of NK cells.

Thus, this study establishes additional mechanisms for the superior proliferation in mbIL21-expanded NK cells, provides a potential approach to increase expansion of NK cells on mbIL15 for adoptive immunotherapy applications, and provides insight into the mechanistic role of miR124-3p in regulating NK cells proliferation and apoptosis.

Table of Contents

Approvals …… i

Title …… ii

Dedication ……. iii

Acknowledgements ……...…… iv

Abstract ……..... vii

Table of Contents..... x

List of Figures xiii

List of Tables …… xv

CHAPTER 1: INTRODUCTION

NK cell Discovery …….. 1

NK Cell Receptors ……. 1

NK Cell Complexity and Functions ……... 4

NK Cell Adoptive Immunotherapy …… 5

Expansion of NK Cells for Adoptive Immunotherapy ……..…….. 7

IL-15 and IL-21 Signaling in NK Cells ……... 11

miRNA: Discovery and Biogenesis ……. 17

miRNA Mechanisms of Gene Regulation …….……...…… 19

miRNA in NK cells: Global Expression and Function …… 21

CHAPTER 2: OBJECTIVES AND STUDY DESIGN

Overall Study Design ……... 24

CHAPTER 3: MATERIALS AND METHODS

Cells and Cell Lines …… 25

NK Cell Isolation and Expansion …… 25

NK Cell Phenotype by Flow Cytometry …… 28

Assessment of Expansion …… 30

NK Cell Cytotoxicity …… 32

Global Expression Analyses …… 33

mRNA Isolation …… 33

Gene Expression Analysis …… 33

Protein Isolation …… 36

Protein Expression Array and Analysis …… 36

miRNA Isolation …… 37

Nanostring Expression Assay …… 37

NK Cell miRNA Expression Analysis …… 38

miRNA Knockdown and Quantitative RT-PCR …… 41

CFSE Proliferation Assay …… 42

Apoptosis Assay …… 42

Gene Expression by qPCR …… 43

Western Blot …… 44

CHAPTER 4: RESULTS AND DISCUSSION

mbIL15 and mbIL21 Lead to Differential Expansion of NK Cells …… 46

NK Expansion on mbIL15 and mbIL21 Differentially Regulate Gene and Protein Expression ……..…….. 53

Gene Expression ……..…….. 54

RPPA Protein Expression ……..…… 66

Expansion Platforms Lead to Differences in miRNA Expression Profiles ……... 76

miR 124-3p ……..…….. 87

Correlation of Key Findings ……..... 88

miR 124-3p Regulates Proliferation, Apoptosis and Senescence in NK Cells …..... 90

miR 124-3p Knockdown Increases Overall Expansion ……... 93

miR 124-3p Knockdown Increases Proliferation ……. 95

miR 124-3p Knockdown Decreases Apoptosis ……..……... 98

miR 124-3p Knockdown Regulates Expression of STAT 3, TERT And BCL2L11 (Bim) …….. 101

CHAPTER 5: SUMMARY AND CONCLUSION …… 107

Impact of Work ……..…… 114

CHAPTER 6: FUTURE DIRECTIONS …… 115

Bibliography ……..……...118

Vita ……. 149

List of Figures

Figure 1: NK Cell Receptors and Secretory Granules ……... 3

Figure 2: Comparison of Fold Expansion of Various NK Cell ex vivo Expansion Methodologies.……... 9

Figure 3: IL-15 and IL-21 Signaling in NK Cells ……13

Figure 4: miRNA Formation and Mechanism of Action …….…… 20

Figure 5: Schematic of NK Cell Expansion and Global Expression Analysis ……..…….. 27

Figure 6: Phenotype of Day 0 NK Cells 29

Figure 7: Verification of Geometric Density of mRNA Expression 35

Figure 8: Schematic for miRNA Data Analysis. …….…… 40

Figure 9: mbIL21 Expanded NK Cells Have Higher Cytotoxicity Potential ……... 48

Figure 10: NK Cells Expanded on mbIL15 And mbIL21 are Phenotypically Similar …..….. 49

Figure 11: mbIL21 Expansion is More Robust and Superior Compared to mbIL15 ..…….... 52

Figure 12: Volcano Plot of mRNA Gene Expression Analysis ……...…… 56

Figure 13: Differential Gene Expression Between mbIL15 and mbIL21 Expanded NK Cells ……... 57

Figure 14: Interrogation of Differential Expressions of Specific Genes Between mbIL15 and mbIL21. …….…... 65

Figure 15: Comparison of Apoptosis Between mbIL15 and mbIL21 NK Cells …….……. 66

Figure 16: Protein Expression Overview in NK Cells, Before and After Expansion …..…… 68

Figure 17: Comparison of Most Differentially Expressed Proteins by RPPA. …..……..…… 71

Figure 18: Expression of Cell Cycle and Apoptotic Proteins in NK cells Before and After Expansion ……...…….. 73

Figure 19: Differential miRNA Expression Between Fresh, mbIL15 and mbIL21 Expanded NK Cells …….……..……...…… 78

Figure 20: Summary of Most Significantly Differential miRNA Between Fresh, mbIL15 and mbIL21 Expanded NK Cells …… 83

Figure 21: Schematic of Correlation of Key Findings miR 124-3p, BCL2L11 and Bim ..…. 89

Figure 22: miR 124-3p Expression in NK Cells Following Knockdown by Electroporation ……... 91

Figure 23: Knockdown of miR 124-3p Enhances Expansion of mbIL15 NK Cells …… 94

Figure 24: Knockdown of miR124-3p Enhances Proliferation in mbIL15 NK Cells …….. 96

Figure 25: Knockdown of miR124-3p Reduces Apoptosis in mbIL15 NK cells ……**....** 99

Figure 26: Knockdown of miR124-3p Increases STAT3 and TERT, and Decreases BCL2L11 in NK Cells ……..…….…… 103

Figure 27: Expansion of NK Cells with mbI15 After Knockdown of miR124-3p Increases STAT3 and TERT, and Decreases BCL2L11 in NK cells …….…… 104

Figure 28: Knockdown of miR 124-3p Increases Stat 3, p-Stat3 and Tert Protein Expression and Decreases Bim Protein Expression ……..……... 106

Figure 29: Schematic of Modified IL-15 Mediated Signaling in NK Cells ……... 110

Figure 30: Schematic of Modified IL-21 Mediated Signaling in NK cells ……..... 111

Figure 31: Schematic of the Effect of miR 124-3p Knockdown on Improving the Overall Expansion Potential of mbIL15 NK Cells …….…… 113

List of Tables

Table 1: Comparison of Features of NK Cells Expanded on mbIL15 And mbIL21 ……..16

Table 2: NK Cell Fold Expansion Calculation ……. 31

Table 3A: Functional Correlation of Highly Expressed Genes in mbIL15 Expanded NK Cells ……..…….. 58

Table 3B: Functional Correlation of Highly Expressed Genes in mbIL21 Expanded NK Cells ……..…….. 59

Table 4: Most Highly Expressed Genes in mbIL15 and mbIL21 Expanded NK Cells and Their Broad Cellular Functions ……. 62

Table 5: Correlation of Differentially Expressed miRNA to Their Known Regulatory Functions ……..……. 85

CHAPTER 1: INTRODUCTION

NK cell Discovery

Natural killer (NK) cells are large granular lymphocytes of innate immune system that play a critical role in host defense against viral infection and surveillance against malignant transformation. NK cells were first identified in 1974 as a subset of lymphocytes that were capable of cell-mediated cytotoxicity against tumor cells, both in cancer patients and animal models. Even in these early experiments, NK cells were observed to cause a 'natural' or 'spontaneous' killing of tumor cells without the need for prior sensitization (1). This phenomenon was later shown in human lymphocyte derived-cytotoxic lymphocytes against human cancer cell lines (2, 3). Later the same year, Heberman et al, isolated 'unique lymphoid cells' from mouse tumor capable of natural cytotoxicity and described them as "Null Cells" since these cells carried no known cell surface markers, and the mechanism of cytotoxicity was distinct from known methods such as antibody dependent cellular cytotoxicity (ADCC) at that time (4). The term "Natural Killer Cells" was coined in 1975 by Keissling et al, to identify a subset of mouse splenic-derived and enriched cells lacking T and B lymphocytes that caused cell mediated lysis against Moloney leukemia cells, and were concluded to be a subset of lymphocytes that were as yet unidentified (5), and later the same year West, et al, identified these cells in human samples (6).

NK Cell Receptors

NK cells are now accepted to be complex in nature, with a large repertoire of activating and inhibitory receptors expressed on their cell surface that drive their intricate anti-tumor functions. It is in fact the expression of these activating and inhibitory receptors that determine

the mode of NK cell cytotoxicity; NK cells do not require priming (prior antigen exposure) and are not restricted by the expression of major histocompatibility complex (MHC) molecules on the target cells, rather the receptors engage a wide variety of molecules on target cell surface (7, 8). It is this feature that also distinguishes NK cells in their selectivity against target cells while sparing 'self' cells. Inhibitory receptors expressed on NK cell surface recognize MHC class I or lack thereof on target cells, thus effectively preventing cytolytic destruction of these cells (9). On the other hand, strong stimulatory signals from activating receptors that overcome inhibitory signals are required for NK cell functioning against the malignant cells. Under normal conditions, lack of stress signals including MHC and absence of stress ligands from infected or tumor cells prevent NK cell activation against 'self'. Tumor and virally infected cells secrete stress ligands that are recognized by NK cells, and tilt the balance toward activation and cytolytic function. Thus maintenance of a critical balance of activation and inhibitory signals on NK cells drive their overall cytolytic potential. NK cells are identified by surface expression of CD56 and CD16, and absence of T cell receptor CD3 ($CD56^{+}CD16^{+}$ $CD3^{-}$) (10, 11). Most peripheral blood NK cells are capable of cytotoxicity in response to simulation by malignant cells (12). Also characteristic of peripheral blood NK cells capable of tumor and viral target recognition and cytotoxicity have high expression of activating natural cytotoxicity receptors NKp46, NKp30 and NKp44 (NCR), with NKp46 expressed on majority of peripheral blood NK cells (13), NKG2D, DNAM1, as well as killer immunoglobulin-like receptors (KIRs) (14). NK cells also express a number of other activating receptors that recognize stress-induced ligands on the surface of the target cell such as NKG2D, NKG2C, CD226 and 2B4 (15). Additionally, other proteins such as CD11a/LFA-1 and TRAIL may play key roles in target cell engagement and enhancing cytotoxicity (Figure 1).

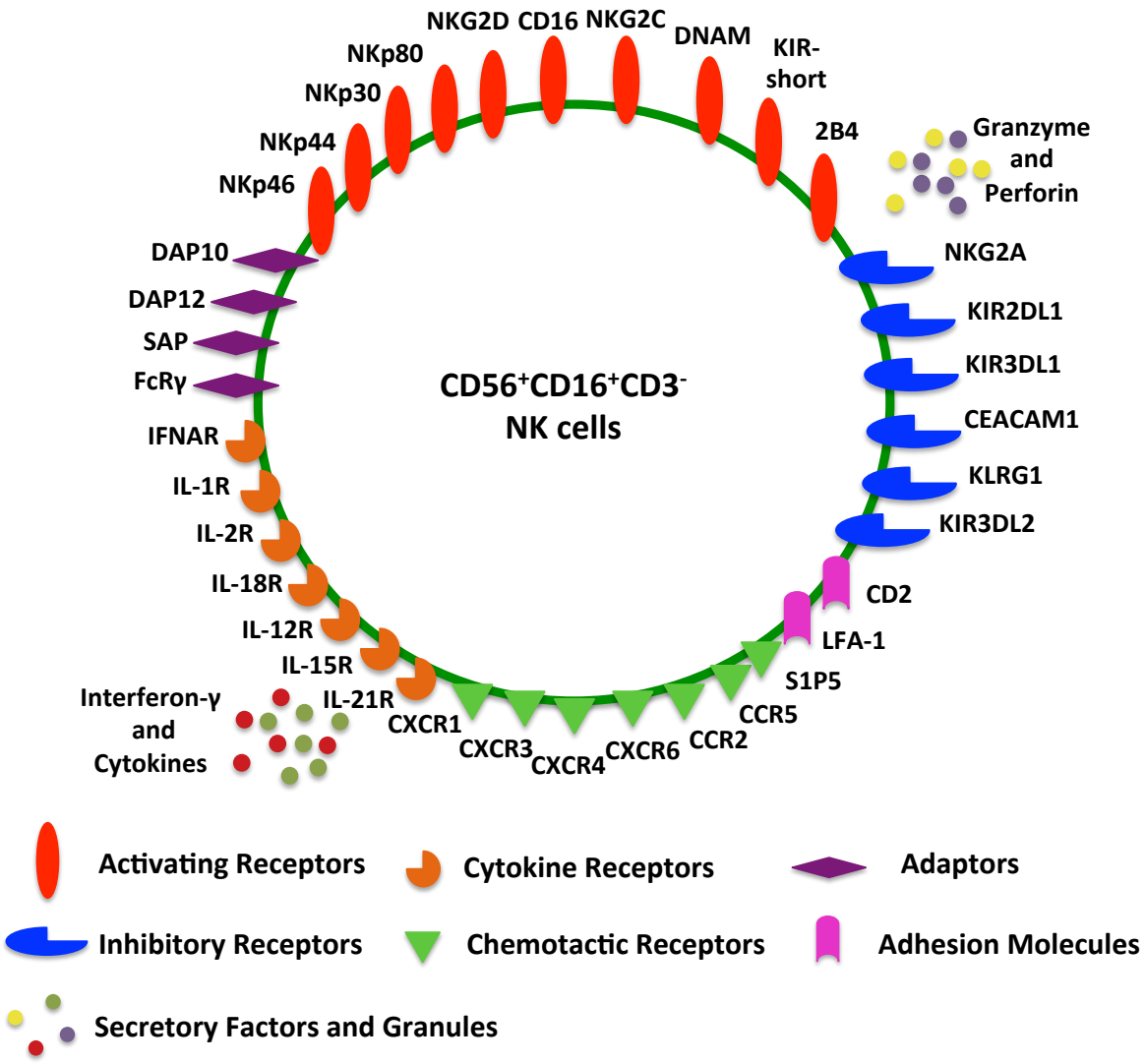


Figure 1: NK cell receptors and secretory granules. NK cells express several cell surface receptors that help maintain balance between the activation and inhibition state of the cells. The receptors shown here are grouped into activating (red), inhibitory (blue), adaptor molecules (purple), cytokine receptors (orange), adhesion factors (magenta), and chemokine receptors (green). Secretory factors such as interferon γ, pro-inflammatory cytokines, perforin and granzyme B are also indicated. This list of receptors, while not exhaustive lends insight into the complexity of NK cells.

NK Cell Complexity and Functions

NK cells are a subset of cytotoxic lymphocytes, and their primary function is in immune surveillance, recognition and killing of virally infected and malignantly transformed cells. These attributes of NK cells are mediated by cytokines as well as the signals generated by activating and inhibitory receptors (16). NK cells mediate their cytotoxic functions primarily through direct lysis of target cells by secretion of lytic granules containing perforin and granzyme, a proteolytic serine protease similar to trypsin (17-19), and by induction of apoptosis of target cells mediated by death receptor pathway proteins such as FAS ligand (FASL), TNF and TRAIL (8, 20). MHC class I molecules are often downregulated in malignant cells. NK cell activating receptors are also capable of recognizing and killing malignant cells' stress induced signals due to cellular stress and DNA damage (21-23). Further, antibody-coated tumor cells or virally infected cells are recognized by FcϒRIII CD16 receptors on NK cells, leading to NK cell activation and resultant release of cytolytic granules and apoptosis of target cells by a process called antibody dependent cellular cytotoxicity (ADCC) (24, 25). NK cells that are highly cytotoxic also secrete a variety of cytokines (26).

Alongside target cell lysis, NK cells also play a role in regulating immune responses of other lymphocytes through secretion of cytokines such as IFN-γ, TNF-α, IL10 and IL13 (27), and the subset of NK cells with cytokine secretory ability are more predominant in lymph nodes and secondary lymphoid organs (28-30). This cytokine secretion ensures the regulation of ADCC as well as activation of pre-existing inflammatory cells, and in recruitment of other immune cells including dendritic, T and B cells. Thus, both innate and adaptive immune responses are mediated by cytokines secreted by NK cells (31-35). Further, depending on

stimulation conditions, peripheral blood NK cell subsets within an individual could be heterogeneous with phenotypically distinct subsets and changes in expression of activating receptors, indicating plasticity and adaptability (36, 37). Additionally, NK cells have recently been shown to exhibit features of adaptive immunity, where they acquire antigen-specific immunological memory similar to T and B cells (38, 39). Further, during hematopoietic stem cell transplantation (HSCT), donor-derived NK cells in T-cell depleted haploidentical grafts were found to exert potent anti-leukemia effects without causing graft *vs* host disease (GvHD) (25, 40-42). These attributes of NK cells have made them an attractive candidate for exploring NK cell adoptive immunotherapy.

NK Cell Adoptive Immunotherapy

Given the diversity of functions of NK cells in cytokine secretion, activation of other immune cell components, lack of ability to cause graft vs host disease, and propensity for cytotoxicity against tumor cells, NK cells have emerged as one of the most promising candidates for adoptive immunotherapy of cancer, and there is significant momentum to advancing NK cells in clinical setting. NK cell therapy is currently being tested against multiple cancers, and adoptive transfer of activated NK cells has been shown to be safe and potentially efficacious for treatment of cancer (24, 43, 44). The therapeutic potential of NK cells has led to several approaches in immunotherapy. Most of the early studies in adoptive therapy were focused on enhancing endogenous NK cells' antitumor capabilities.

Some of the early trials included induction of autologous NK cells by systemic administration of IL-2 against breast cancer and lymphoma. However, lower doses did not

potentiate substantial activation and cytotoxic function, while higher doses caused an increase in systemic cytokines with toxicities associated with high dose IL-2 in patients (40, 45, 46). Similarly, activation of NK cells with other cytokines like IL-12, IL-15, IL-18, IL-21 and type I IFNs yielded moderate generation of NK cells, but not sufficient to cause clinical responses (47). In clinical setting, autologous NK cell therapy using IL-2 activated NK cells were found to be safe but less efficacious, and was attributed to lack of KIR mismatch with autologous tumor cells (48-52). In order to overcome this drawback, IL-2 activated allogeneic NK cells were used in AML patients, but with limited success (43). NK cells that are activated and expanded ex vivo prior to adoptive transfer using "cytokine cocktail" with soluble cytokines such as IL-2, IL-15 and IL-21 have also been tested in clinical trials with limited success (53-57), partly due to the number of NK cells generated by these methods are much lower than an 'ideal' number to cause a favorable clinical outcome.

Despite these significant advances in NK cell adoptive immunotherapy, the bottleneck for success has been finding methods of generating large number of NK cells with high purity and potency required for therapeutic benefit. Most cancer clinical trials require NK cells ranging from 5×10^6 to 5×10^7 per kilogram (43, 58-60), with recent studies using doses as high as 1×10^8 NK cells per kilogram per infusion. Given the small percentage of NK cells in peripheral blood (61, 62), the amount of NK cells required for infusion into patients become challenging. Several attempts have focused on finding robust methods of ex vivo expansion of NK cells. Expansion of NK cells from sources such as embryonic stem cells, cord blood, and buffy coats (peripheral blood monocytic cells, PBMCs) have opened up possibilities of increased expansion and a shift in NK cell adoptive immunotherapies. Some of the ex vivo expansion methods for NK cells are discussed in the section below.

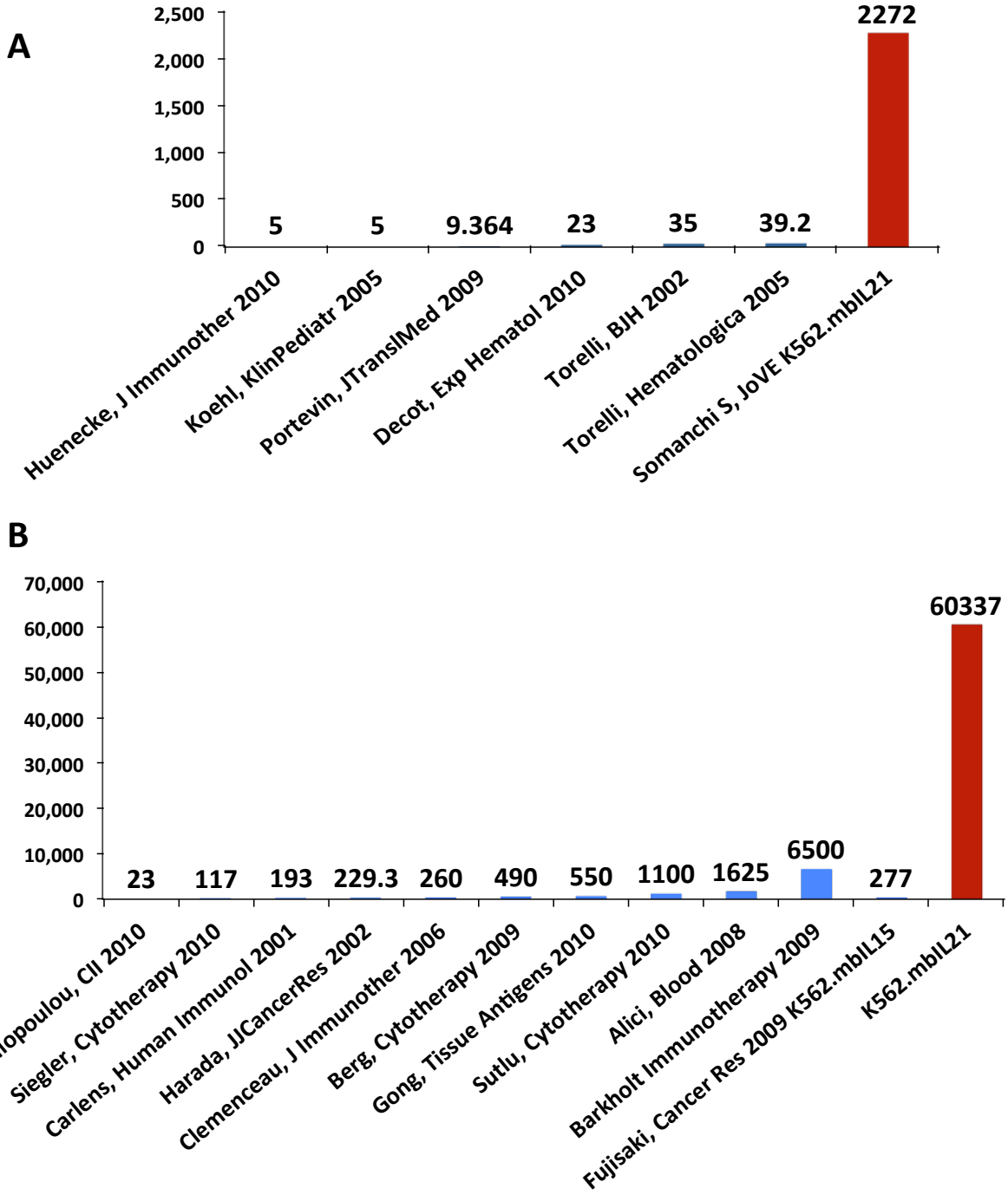
Expansion of NK Cells for Adoptive Immunotherapy

One of the early methods for isolation of peripheral blood NK cells for immunotherapy was the traditional steady state leukopheresis (followed by T cell depletion and overnight activation with IL-2). This method is not only cumbersome but also yielded very low numbers of NK cells for patient infusions during clinical trials, due to the lower percent of NK cells in the peripheral blood. A typical leukopheresis isolation of NK cells yielded less than 2 X 10^7 cells for a single infusion (63). Magnetic selection of NK cells followed by activation with high dose IL-2 and IL-15 yielded moderate expansion of $CD56^+CD16^+CD3^-$ NK cells, but did not eliminate $CD56^+CD16^+CD3^+$ cells (52). Expansion platforms using bisphosphonate-coated dendrimers for selective expansion of NK cells from PBMCs (64), feeder cells derived from EBV-lymphoblastoid cell lines (LCL), achieving a mean 490-fold expansion in 21 days have also been reported. These cells were tested successfully against adult and pediatric leukemia in clinic (65-67). K562 expressing 4-1BBL and MIC-A, plus soluble IL-15 was shown to yield 550-fold expansion of NK cells in 24 days (68). Overall, these methods enabled ex vivo expansion of NK cells from ~35 folds in (12 days) to 1625 folds (2 weeks) (69) (Figure 2).

Prominent amongst the various expansion platforms include K562-based feeder cells developed by transducing 4-1BBL (CD137L) and membrane-bound IL-15 (K562.mbIL15) (70, 71), and K562-based feeder cells transduced with CD64, CD86, CD137L, truncated CD19 and membrane bound IL-21 (K562.mbIL21) developed in our lab (62, 69, 72). K562.mbIL21 surpasses any other expansion to date not only in its rate of proliferation, but also cytokine secretion, cytotoxicity, telomere length and MTOC synapse formation, yielding highly activated non-senescent cells, with an average 67,000 fold expansion of pure NK cells in 21

days. Both K562.mbIL15 and K562.mbIL21 circumvent the high toxicities involved with soluble cytokines and cytokine cocktails by the use of membrane bound versions of IL-15 and IL-21, respectively. A comparative fold expansion of various methods published thus far ranging from 12 days to 21 days is represented in Figure 2.

NK cells expanded on K562.mbIL15 yields a mean expansion of 277 folds in 21 days, and although the cells are highly cytotoxic both in vitro and in vivo, their proliferation is limited due to senescence, attributed to telomere shortening (70). NK cells expanded on these platforms are currently used in several clinical trials (K562.mbIL15: *NCT02123836, NCT03003728*; K562.mbIL21: *NCT02809092, NCT01787474, NCT02005289, NCT00514085*). Additionally, our K562.mbIL21 expansion methodology is widely used to study several aspects of NK cells including NK cell biology (73, 74), expansion of NK cells form umbilical cord blood (75), human embryonic stem cells and inducible pluripotent stem cells (76, 77), preclinical evaluation of neuroblastoma (78, 79), as well as expansion from rhesus macaques and canine models (80, 81).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Gong, Tissue Antigens 2010
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Fujisaki, Cancer Res 2009 K562.mbIL15
K562.mbIL21

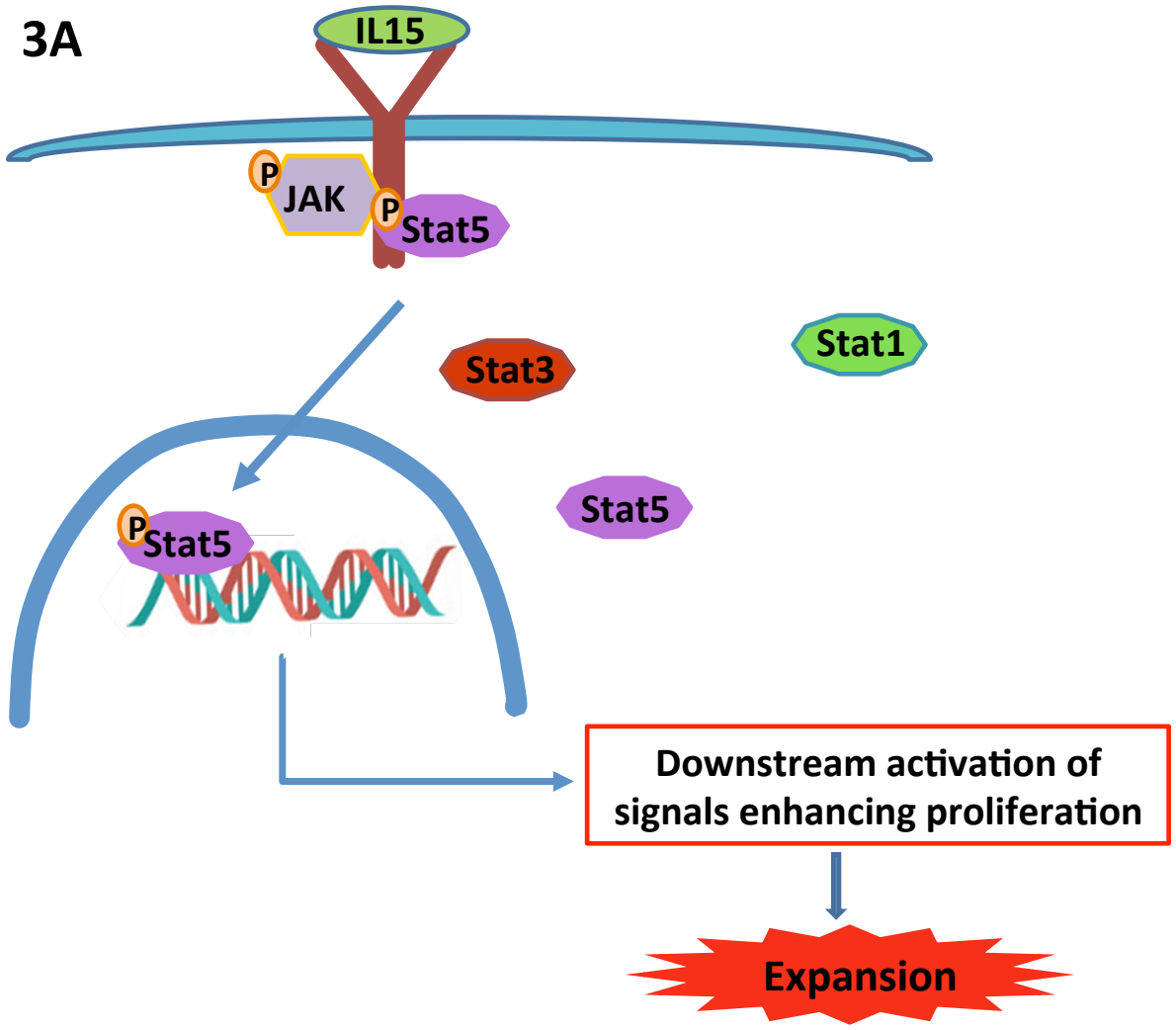
Figure 2: Comparison of fold expansion of various NK cell ex vivo expansion methodologies. (A) Methods of NK expansion in this panel include high dose IL-2 cytokine stimulation, leukopheresis, dendrimer-based expansion platform, cytokine cocktails and membrane bound IL-21 from left to right. Of the expansion methods for 2 weeks included in this panel, only mbIL21 expansion yields highly pure NK cell product without contaminating CD3 positive cells, and circumvents toxicities associated with soluble cytokines. (B) This panel includes methodologies where NK cells were expanded for 21 days or longer. Methods include soluble cytokines IL-2, IL-15 in combination with other agents such as hydrocortisone, anti-CD3 antibody, OKT3, K562 cell line stimulation; EBV transformed LCL cell line, K562 gene modified with membrane bound IL-15 and K562 gene modified with membrane bound IL-21. As shown, mbIL21 expansion surpasses other methods by several thousand folds, while making the NK cells highly potent and cytotoxic against a wide variety of tumors tested.

IL-15 and IL-21 Signaling in NK Cells

K562.mbIL15 and K562.mbIL121 platforms rely on IL-15 and IL-21 cytokine signaling respectively. Both cytokines' signaling activation is via JAK-STAT pathway, however they differ in their specific pathway of action. IL-15 and IL-21 *preferentially* and predominantly activate through signal transducer and activator of transcription – STAT 5 and STAT 3, respectively (82). IL-15 and IL-21 belong to the class of common γ-chain cytokines, along with IL-2. IL-15 has long been recognized for its role in in vivo lymphocyte activation, maturation, proliferation and homeostasis, particularly in NK cells (83, 84). In vivo, IL-15 is considered a pivotal cytokine required for NK cell development and maintenance. Signaling is initiated when IL-15 binds to its cognate receptor IL-2/15Rβγ on NK cells, and activates Janus-associated kinase 3 (JAK3). This series of events preferentially activates phosphorylation of STAT5 via IL-2/15Rβγ heterodimer's γ-chain (85), promotes translocation to the nucleus and binding to specific DNA elements causing activation of downstream signals, thus enhancing proliferation and cytotoxic effector functions (86-88) (Figure 3A). Similar to IL-15, activation of signaling by IL-21 is initiated when IL-21 binds to IL-2/21Rβγ receptor on NK cells, however it activates Janus-associated kinase 1 (JAK1) (89, 90). This in turn preferentially recruits and activates STAT3 phosphorylation and activates further downstream signaling leading to proliferation as well as enhancing cytotoxicity properties of NK cells by activation of granzyme B and perforin (Figure 3B). Interestingly, IL-21 is a pleiotropic cytokine and its function varies from one immune cell type to the other (91). IL-21 signaling in NK cells in synergy with IL-15 and IL-18 has been shown to promote proliferation, maturation, increase interferon gamma (IFN-γ) production, and increase cytotoxicity of NK cells in vivo (92, 93). Additionally, whilst IL-21 signaling is highly

beneficial to the immune components like CD8 T cells, dendritic cells, macrophages, and particularly NK cells, as well as enhancing NK cell mediated ADCC, it has been shown to induce apoptosis in certain lymphomas and other malignancies (94-97). Based on these attributes of IL-21 signaling and advantages, we developed the K562 feeder cell based mbIL21 platform for the expansion of NK cells.

In our studies, we have previously extensively compared the properties of NK cells expanded on K562.mbIL15 and K562.mbIL21 (mbIL15 and mbIL21, respectively), since both expansion platforms are based on feeder cells modified to express membrane bound cytokines (Table 1), we showed that there are certain similarities as well as differences between the NK cells expanded on mbIL15 and mbIL21. For instance, both mbIL15 and mbIL21 expanded NK cells had similar phenotypic expression of activating and inhibitory receptors, and their effect on cytotoxicity, with marginal differences in these attributes (62). In their original report of mbIL15 feeder cell expansion platform for NK cells, Fujisaki, et al demonstrated that mbIL15 causes decrease in telomerase reverse transcriptase enzyme (hTERT), and consequently replicative senescence in NK cells. This effect was overcome by the addition of recombinant hTERT (70). On the other hand, STAT3 is a known activator of hTERT (98), and we reported that mbIL21 expanded NK cells indeed had significantly higher telomere length. The biggest observed difference however was in the fold expansion between mbIL15 and mbIL21, as shown in Figure 2 and Table 1. It is possible that mbIL21 expansion is significantly better due to the regulation of telomere length by promotion of telomerase activity, however this may not be the only determining factor.


3A
IL15
P
JAK
P
Stat5
Stat3
Stat1
Stat5
P
Stat5
Downstream activation of signals enhancing proliferation
Expansion

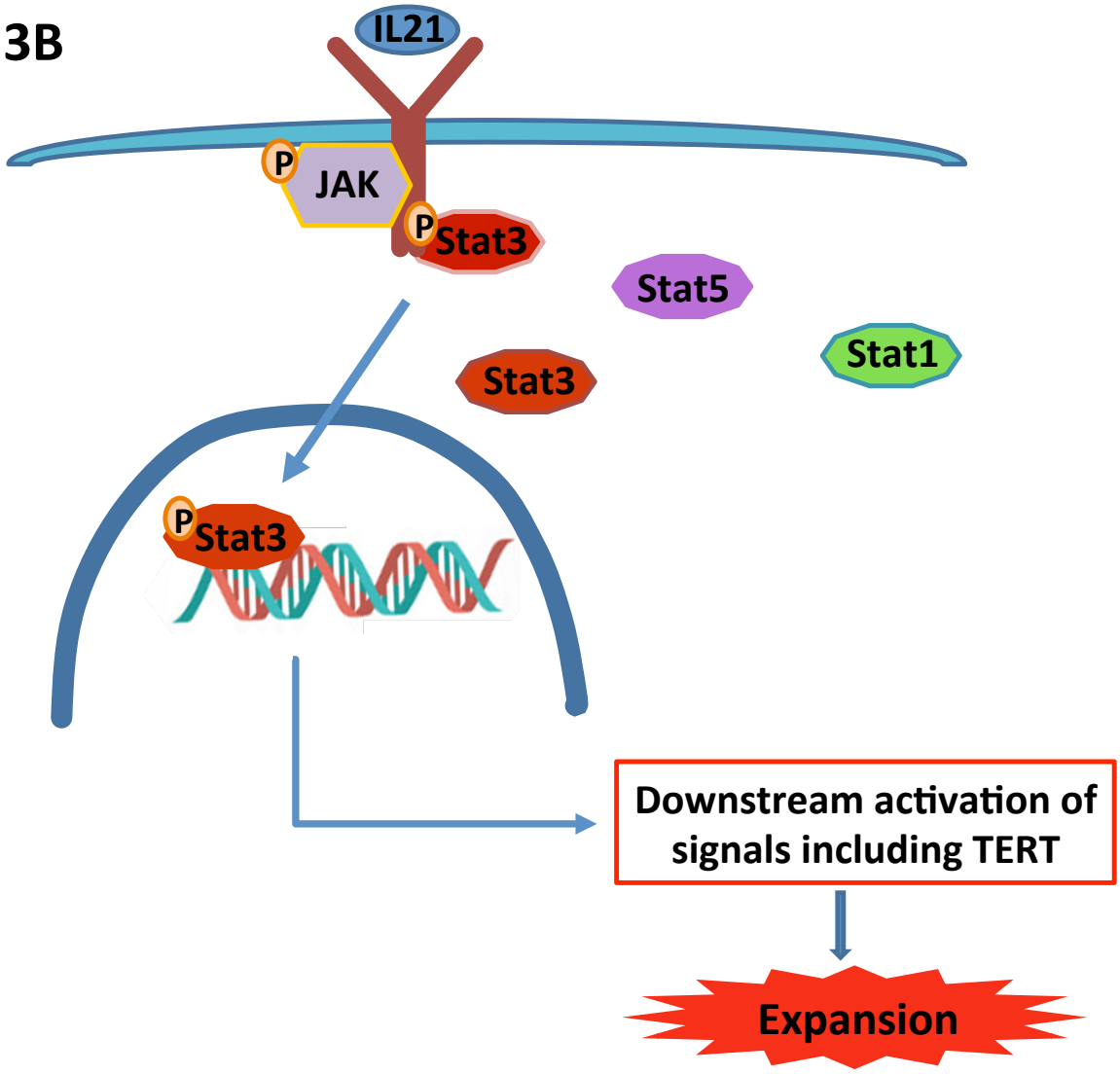

3B
IL21
P
JAK
P
Stat3
Stat5
Stat1
Stat3
P
Stat3
Downstream activation of signals including TERT
Expansion

Figure 3: IL-15 and IL-21 signaling in NK cells. (A) IL-15 binding to its cognate receptor IL-2/15Rβγ activates Janus Kinase (JAK) phosphorylation, which leads to recruitment, and preferential phosphorylation and activation of Stat5. The now activated Stat5 is translocated to the nucleus where it binds specific DNA elements causing activation of downstream signals, including PI3K, MAPK pathways thus enhancing proliferation and cytotoxic effector functions in NK cells. When NK cell stimulation occurs in vivo in response to cellular stress, IL-15 mediated stimulation leads to active proliferation of NK cells by prevention of apoptotic signals. This, in addition to the well-established role of IL-15 in activation, proliferation and homeostasis in vivo, was the basis upon which mbIL15 expansion platform was developed. (B) Similar to IL-15, IL-21 binding to the IL-2/21Rβγ promotes preferential recruitment and phosphorylation of Stat3, which in turn is translocated to the nucleus, where it binds the DNA elements and causes activation of downstream signals including PI3K, MAPK, BCL-2 (anti-apoptotic) and granzyme A, thus enhancing proliferation and cytotoxic effector functions in NK cells. IL-21 is a pleiotropic cytokine with varied functions in different immune cell types, and in vivo IL-21 mediated signaling in NK cells promotes proliferation, maturation, survival, increased interferon gamma (IFN-γ) production, and increased cytotoxicity.

	Expansion based on gene modified feeder cells	
	K562.mbIL15	K562.mbIL21
Phenotype	High Expression of Activating Receptors	High Expression of Activating Receptors
Cytotoxicity	High Potency	High Potency (greater than mbIL15 expanded NK cells)
Clinical Trials	Yes	Yes
Activation Signal	Preferentially Through STAT 5	Preferentially Through STAT 3
Telomere Length	Low - Senescent NK cells	High - Non-senescent NK cells
Expansion (3 weeks)	277 fold	67000 fold

Table 1: Comparison of features of NK cells expanded on mbIL15 and mbIL21. Based on our previous studies, we compared the properties of NK cells expanded on mbIL15 and mbIL21. Both expansion platforms are based on feeder cells modified to express membrane bound cytokines. As shown, phenotype and cytotoxicity were similar in our prior studies. The differences include activation signaling through Stat 5 and Stat 3 for IL-15 and IL-21, respectively. Additionally, mbIL21 expansion promoted maintenance of telomere length, whereas mbIL15 expansion diminished TERT expression, leading to senescent cells. The largest and the most significant difference between the expansion methods were in the fold expansion after 3 weeks, as shown.

Although a number of transcription factors and molecular mechanisms regulating various aspects of NK cells including their development, maturation, survival, and function have been identified, several of the regulatory aspects of NK cells are under active investigation. In this project, we sought to address the key differences in expansion between mbIL15 and mbIL21 and the mechanisms involved in causing their expansion outcome. We proposed that the robustness in mbIL21 expansion compared to mbIL15 could be due to an increased proliferation of NK cells with mbIL21, increased senescence and apoptosis in mbIL15 expansion or a combination of all these factors. We also proposed that genes and proteins involved in regulation of cellular proliferation, apoptosis and senescence could be differentially expressed in mbIL15 and mbIL21 expanded NK cells. Further, these differences could have basis in differential transcriptional and post-transcriptional regulation of gene expression, and could be mediated and regulated through the involvement of microRNA (miRNA).

miRNA: Discovery and Biogenesis

MicroRNA (miRNAs) are a large class of small non-coding RNA molecules that are 18-22 nucleotides long and are highly conserved across species. miRNAs were discovered in 1993; they comprise 3-4% of human genome and are concentrated in and around tumor susceptibility loci (99, 100). miRNAs act as key regulators of post transcriptional gene expression, and regulate over 30% of known mammalian genes to date (101). More than 2500 miRNAs have been discovered, and they control complex regulatory network of gene expression. miRNA mediated regulation has been observed in several signaling and cellular processes including tissue development, survival, cell differentiation, proliferation, neuronal

development, metabolism, stem cell maintenance, apoptosis and immune modulation (102-110). Deregulation of miRNA expression is commonly found in cancers, and these changes are implicated in tumorigenesis, invasion, proliferation and increased metastasis (111); several miRNAs are currently used as diagnostic biomarkers. Modulation and restoration of miRNA expression levels using miRNA inhibitors and/or mimetics are being investigated as therapeutic intervention against several cancer models. (112-114).

The miRNA are formed from long non-coding RNAs through a series of enzymatic steps. The sequential processing of miRNA occurs fist in the nucleus followed by the cytoplasm, where they bind to genes and regulate gene expression by either suppression or activation of cognate mRNA. miRNA genes are transcribed in the nucleus as pri- and pre-double stranded miRNA; the pri-miRNA is processed in the nucleus by the RNAse enzyme Drosha into a 70 nucleotide-long hairpin-like precursor called pre-miRNA, which is exported to the cytoplasm by exportin 5-Ras GTPase complex. In the cytoplasm, pre-miRNA undergoes additional processing by another RNAse III enzyme Dicer, thus generating the double strand miRNA (115-117) (Figure 4).

The transcriptional regulation action of miRNA is mediated through base-pairing between the double strand miRNA – Argonaute protein complex (Ago-1 and Ago-2) and an imperfectly matched target site usually located at the 3′-end of a target mRNA. This process leads to unwinding and elimination of the passenger strand, leaving the mature miRNA to be incorporated into the RNA-inducing silencing complex (RISC), resulting in translational repression followed by deadenylation and degradation of the mRNA (Figure 4). Each miRNA has been shown to affect several of its target mRNA in a given cell, and the strength of

repression or activation is dependent on the binding affinity of the miRNA-mRNA-RISC complex (118, 119).

miRNA Mechanisms of Gene Regulation

Although traditional roles of miRNA in gene regulation were considered to be translational repression, recent studies show evidence of overall gene regulation by repression as well as activation of protein expression (111). The miRNA-mediated downregulation has been shown to be reversible. Additionally, it has also been observed that miRNA upregulates certain proteins in specific cell types and conditions, in response to cellular stimuli (120-122).

The miRNA suppress gene expression by several means: shortening of poly A tail, cleavage and destruction of mRNA, destabilization of mRNA (partial cleavage), inhibition of translational initiation and/or elongation, as well as ribosomal obstruction of translation leading to lower output of target proteins (123, 124). In order to cause downregulation of gene expression, each miRNA pairs with the 3′ untranslated region (3’ UTR) of its mRNA target mediated by a 6–8-nucleotide seed sequence at the 5′ end of the miRNA, which in turn binds the 3’ UTR of mRNA promoter region (125). Some miRNAs have also been shown to target 5’ UTR of mRNA, the coding regions of mRNA (120, 126) (Figure 4).

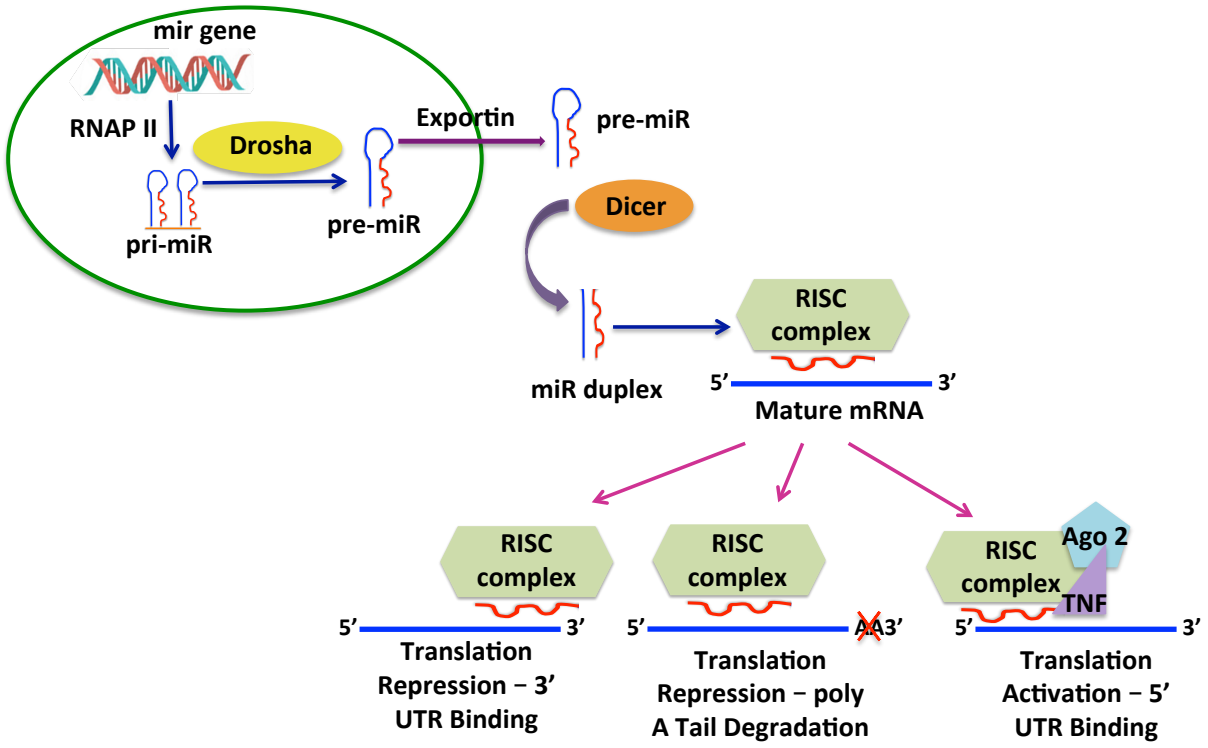


Figure 4: miRNA formation and mechanism of action. The formation of functionally active miRNA is an enzymatically regulated process that begins in the nucleus with miR gene transcribed into pri-miR, which by a series of steps involving Drosha and Dicer enzyme complexes leads to the formation of a mature 'sense' strand of miRNA in the cytoplasm. This sense strand forms a RNA-Induced Silencing Complex (RISC), and binds mature mRNA. The effector function of miRNA regulation is determined by binding of miRNA on specific translational activation or repression domains of mRNA. 3' UTR mRNA-miRNA binding cause translational repression as shown, while incomplete binding causes poly A tail degradation, and thereby repression. Recent studies have shown 5' UTR mRNA-miRNA binding specifically recruits TNF and Ago 2 proteins leading to translational activation.

The mechanisms that drive upregulation of protein expression due to miRNA intervention are diverse as well. For example, a recent study showed that when Ago2-RISC complex binds to Fragile X mental retardation protein 1 (FXR1) protein, it leads to direct activation of eIF4E (elongation factor), and thereby translation (127). A similar mechanism was reported when miR 369-3 interacted with AU rich region of tumor necrosis factor (TNF) mRNA, and caused augmentation of protein expression during cell cycle arrest. Specifically, direct base pairing between the miR 369-3 and its target caused translational upregulation after cellular stress stimuli such as serum starvation (120, 128). In the same study, miR let-7a was shown to switch the target genes from translational repression to activation during cell cycle arrest. miRNA binding to 5' UTR region has been associated with enhancing ribosomal biogenesis, causing increased global protein synthesis and subsequent oncogenic transformation in NIH3T3 cells (129). Additionally, AGO2 has been shown to be involved in activation of gene expression when the target mRNA lacks poly(A) tail (130). Thus, miRNA regulation of gene expression is through diverse mechanisms of both translational repression and activation.

miRNA in NK cells: Global Expression and Function

In literature, the role of miRNA is extensively studied in various cellular functions such as proliferation, apoptosis and migration in several cancers, immune cells such as T cells, and to a smaller extent in NK cells. Recent advances have highlighted the importance of miRNA-mediated post-transcriptional regulation in NK cell development, maturation, and function (131-133). Several studies on miRNA in NK cells are focused on global expression

and observational analyses of miRNAs. It is conceivable that the expression profiles of miRNA in NK cells would be determined by the source and mode of activation of NK cells. Thus, there are several studies that have explored miRNA expression in NK cells form mouse (132), cord blood (134), NK-92 cell line (135) and NK cells stimulated with cocktail of soluble IL-2, IL-15 and IL-21 cytokines (136). Whilst this is a growing field, there are however, currently no reports of comparison of miRNAs in membrane bound IL-15 and IL-21 expanded NK cells or the role of miRNA in promoting robust expansion of NK cells with IL-21. This aspect of the study is of high interest to us, particularly because this platform was developed in our group, it indeed is better than all other platforms for ex vivo expansion of NK cells developed thus far, and that NK cells expanded on mbIL21 are currently in several clinical trials. Hence we proposed that *activating and expanding human NK cells on membrane bound IL15 and membrane bound IL21 will lead to differential gene expression mediated by miRNAs and subsequent observed differential expansion potential in these cells.*

Further, there are currently several reports where the roles of specific miRNAs in NK cell function have been shown. Most of these studies focus on delineating the role of miRNA in modulating NK cell development, maturation and function (133, 137-141). There are currently no studies on the roles of specific miRNA in regulating cytokine-mediated proliferation and expansion of NK cells. Thus the overarching goal of this project was to identify the differential gene expression, and if stimulation of NK cells with mbIL15 and mbIL21 could differentially regulate miRNA profiles of these cells, and if so how these miRNA influence the observed difference in expansion of NK cells. It is worthwhile to note that while we have specifically focused on the role of miRNA in regulating expansion aspect of NK cells, we have also identified miRNA that has potential to modulate other aspects of NK

cells function including cytokine secretion, chemokines and chemokine receptor expression, expression of activating and inhibitory receptors and intracellular granzyme B and perforin expression, as well as NK cell development. These miRNAs are discussed in the future directions section of the thesis, with potential future studies including modulation of these miRNA either during expansion on mbIL21 or to study NK cell biology.

CHAPTER 2: OBJECTIVES AND STUDY DESIGN

Based on the rationale provided above, the overarching objectives for this study are to (1) understand the global miRNA, gene and protein expression profiles of NK cells that are isolated from peripheral blood, as well as NK cells that are expanded on K562.mbIL15 and K562.mbIL21 feeder cells, (2) to identify the differential expression of miRNA, gene and proteins in K562.mbIL15 and K562.mbIL21 expanded NK cells, (3) to correlate the differentially expressed miRNA, gene and proteins that play a role in determining the observed changes in expansion, and (4) to validate the identified miRNA targets in these NK cells by modulating their expression through transient knockdown/ upregulation by electroporation.

Overall Study Design

The differential global expressions of miRNA, gene and protein were assessed, followed by statistical analyses and correlation of miRNA, gene and protein expressions. miR 124-3p, *BCL2L11* and Bim were identified to be the most significantly differentially expressed factors with highest expression in mbIL15 expanded and fresh NK cells. We further correlated miR 124-3p to *BCL2L11* as well as other genes that are critical to the expansion outcome. In order to understand the role of miR 124-3p in expansion of NK cells, miR124-3p was knocked down in NK cells and evaluated for its effect on regulating proliferation, apoptosis and senescence as key functions that cause the overall observed differential expansion.

CHAPTER 3: MATERIALS AND METHODS

Cells and Cell lines

Anonymized normal donor derived buffy coats were obtained form Gulf Coast Regional Blood Center (under IRB approved protocol) and the MDACC blood bank. Peripheral blood mononuclear cells (PBMCs) were isolated from these buffy coats by ficoll paque premium plus (GE Healthcare, USA) centrifugation as described previously (62, 69, 72). NK cells were isolated from the separated PBMCs as described below.

CML cell line K562 was purchased from ATCC, AML cell line Molm 13 was a gift from Dr. Zweidler McKay's lab, Division of Pediatrics, MDACC. Both cell lines were maintained in RPMI 1640 containing 10% FBS, 1% L-Glutamine and 1% penicillin/ streptomycin. The authenticity of the cell lines was confirmed by short tandem repeat DNA profiling analysis (Promega Powerplex 16HS Kit).

NK Cell Isolation And Expansion

NK cells were isolated from PBMCs by negative selection with RosetteSep Human NK cell enrichment Cocktail (Stem Cell technologies, Ca), and the cells were maintained in complete RPMI 1640 (Cellgro, VA) containing 10% Fetal Bovine Serum (Invitrogen, CA), L-glutamine (Gibco, CA), penicillin/streptomycin, sodium pyruvate and non-essential amino acids (Cellgro, VA) and freshly supplemented with 50 IU/ml recombinant human IL-2

(Proleukin, Novartis) [1, 3]. Freshly isolated NK cells were expanded by stimulation with gamma-irradiated (100 Gy) feeder cells K562.mbIL15 or K562.mbIL21 (for each donor) [3] for 2 weeks (14 days) by co-culture at 2:1 ratio in complete RPMI media. Half of the media was replaced every 2 days or as needed and supplemented with fresh IL-2 for the entire volume of media. At the end of 1 week of expansion (Stim 1), NK cells were again co-cultured with 1:1 ratio of gamma-irradiated feeder cells in complete RPMI media freshly supplemented with 50 U/ml IL-2 (stim 2); NK cells were collected at the end of 14 days (Figure 5).

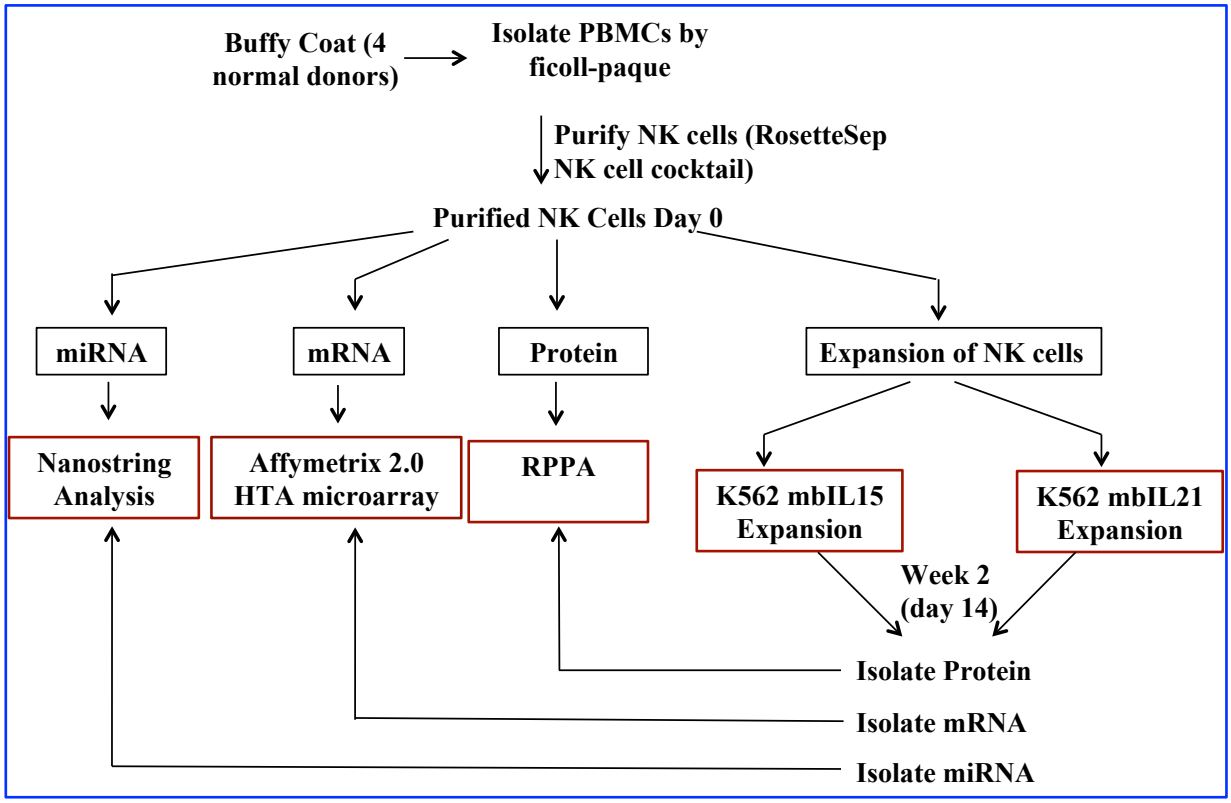


Figure 5: Schematic of NK cell expansion and global expression analysis. NK cells from 4 normal donors were isolated using Rosettesep NK cell cocktail, and miRNA, mRNA and protein were isolated from these cells as shown. 2 aliquots of these NK cells each were stimulated with either K562.mbIL15 or K562.mbIL21 for 2 weeks. NK cells were checked for purity after 2 weeks by flow cytometry, and miRNA, mRNA and proteins were isolated from these samples as before. As indicated, miRNA analysis was performed by Nanostring analysis, mRNA by Affymetrix HTA 2.0 and protein by RPPA.

NK Cell Phenotype By Flow Cytometry

Freshly isolated (unexpanded) NK cells were stained with anti-CD56, anti-CD16, anti-CD3 and anti-NKp46 antibodies (BD Biosciences, USA) and assessed by flow cytometry using BD FACSCalibur. Data was analyzed with FlowJo Version 10 software (TreeStar Inc) and GraphPad Prism. NK cells that were >99.9 % $CD3^{neg}CD56^{pos}$ $CD16^{pos}$ $NKp46^{pos}$ were considered pure and used for further studies (Figure 6).

Phenotype of expanded NK cells was assessed based on the expression of a panel of surface markers. Fluorochrome conjugated antibodies were used to assess the expression of anti-CD56, Anti-CD16, anti-CD3, anti-NKp46, anti-NKp44, anti-NKp30, anti-NKG2D, anti-CD244, anti-CD226, anti- CD11b, anti-CD27, antiCD11a, anti-TRAIL, anti-CD28, anti-CD2, anti-CD57, anti-CD62L, anti-CD69, anti-CD25 (BD Biosciences, USA), anti-CD160 (Biolegend, USA), anti-KIR2DL1/DS1, anti-KIR 2 DL2/3, anti-KIR3DL1 (Miltenyi, USA), anti-KIR3DL1, anti-NKG2A and anti-KIR2D1 (R&D Biosystems, USA).

Briefly, a small aliquot of rosette sep purified NK cells (0.5E+05 cells) were resuspended in blocking medium (50% FBS in PBS) and incubated for 30 minutes at 4 °C. Following this, NK cells were stained with antibody cocktails of the cell surface markers described above (69), and assessed by flow cytometry using BD FACSCalibur and data was analyzed with FlowJo Version 10 software (TreeStar Inc). Student t-test, and one-way ANOVA were used to statistically analyze the combined data using Graphpad Prism Version 6 and $p < 0.05$ was considered significant.

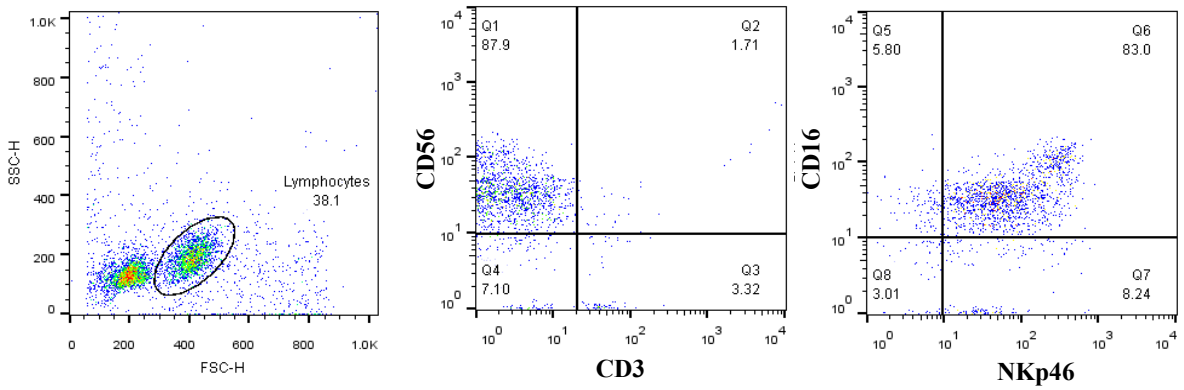


Figure 6: Phenotype of day 0 NK cells. NK cells were isolated from normal donor-derived buffy coats by rosette sep purification. Purity of NK cells was assessed by flow cytometry. NK cells were stained with anti-CD56 FITC, anti-CD16 PE, anti-CD 3 PeCy5 and anti-NKp46 APC. In order to prevent expression analyses miRNA, gene and protein expression of contaminating T cells, only NK cells with < 1% of CD3 positive cells were considered for further experiments. However, if NK cells were only to be used for expansion purposes, presence of contaminating T cells in the culture would be eventually eliminated after 2 weeks of expansion. We have previously shown that pure population of NK cells is achievable even when we start the expansion cultures with PBMCs. Data shown here is representative from 1 donor.

Assessment Of Expansion

The fold expansion of NK cells on mbIL15 and mbIL21 was calculated as shown in Table 2. Briefly, NK cell expansion was started with > 99.9% $CD3^{neg}CD56^{pos}CD16^{pos}$ $NKp46^{pos}$ cells, and the fold expansion was calculated based on this population. The overall fold expansion for each week was calculated using the formula:

$$\frac{\text{Inferred Total NK cells expanded/week } \mathbf{X} \text{ total number of cells in culture}}{\text{NK cells carried forward for expansion each week.}}$$

When all NK cells are not carried forward for expansion, the data is presented as "Inferred fold expansion" calculated based on the formula:

$$\frac{\text{Inferred total NK cells after } \mathbf{`x'} \text{ weeks of expansion}}{\text{Inferred total NK cells after week 1 of expansion.}}$$

where 'x' is the number of weeks (in this case 2 weeks). An example of fold expansion calculation is included in the table below. The differential fold expansion between mbIL15 and mbIL21 expanded NK cells was statistically analyzed by paired student t-test, using Graphpad Prism Version 6 and $p < 0.05$ were considered significant. Fold expansion of NK cells following knockdown of miR 124-3p was calculated in a similar manner.

		A	B	C	D	E	F	G
1		**Day 0 Starting NK cell number**	**NK cell purity (%)**	**Day 0 Actual NK numbers**	**Day 7 Cell Count**	**% NK cells**	**Day 7 Actual NK cell numbers**	**Weekly fold expansion**
2	**Calculation**	Actual number of NK cells derived from buffy coat	100 (since these are rosette sep purified)	A2*B2/100	Raw cell counts on day 7	100 (since these are rosette sep purified)	D2*E2/100	F2/C2
3	**Example**	5,000,000	100	5,000,000	95,000,000	100	95,000,000	19

Continued below…

		H	I	J	K	L	M	N
1		**Carried Forward for 2nd week of expansion**	**Day 14 cell Count**	**% NK cells**	**Day 14 Actual NK cell numbers**	**Weekly fold expansion**	**Inferred overall NK cell numbers**	**inferred overall expansion fold**
2	**Calculation**	Desired amount of NK cells	Raw cell counts on day 14	100	I2*J2/100	K2/H2	L2*F2	M2/C2
3	**Example**	5,000,000	1,250,000,000	100	1,250,000,000	250	2.375E+10	**4750**

Table 2: NK cell Fold Expansion Calculation. Rosette sep purified NK cells from 4 donors were expanded on K562.mbIL15 or K562.mbIL21 for the initial expression analysis studies. Cells were expanded for 2 weeks and fold expansion was calculated as shown. Data shown here is an example. For knockdown studies, unelectroprated control NK cells, scrambled negative control and miR 124-3p knocked down NK cells were expanded on mbIL15 or mbIL21 simultaneously, and fold expansion was calculated from 5 experiments.

NK Cell Cytotoxicity

Cytotoxicity mediated by expanded NK cells was assessed by calcein release assay as previously described (69, 142). Target cell lines K562 and Molm13 were stained with 2 μg/ml Calcein AM (Life Technologies, Carlsbad, CA) in complete RPMI media containing 1 mg/ml stock of calcein AM (staining media). $1x10^6$/ml tumor cells were resuspended in staining media and incubated for 30 minutes at 37°C in 5% CO2 incubator with intermittent mixing, then washed and resuspended at $1x10^5$ cells/ml. In the meantime, mbIL15 and mbIL21 expanded NK cells were counted, and resuspended at $1x10^6$ cells/ml. NK cells were then two fold serially diluted 5 times in a U-bottom 96 well plate. All experiments were set up in triplicates. The final NK cell numbers in each triplicate set were $1x10^5$, $5x10^4$, $2.5x10^4$, $1.25x10^4$, $6.25x10^3$ and $3.125x10^3$ cells/well. Calcein-AM loaded target tumor cells were added to each of these wells at $1x10^4$ cells/well in 100-μl volumes for final E:T ratios of 10:1, 5:1. 2.5:1, 1.25:1, 0.625:1 and 0.3125:1. $1x10^4$ calcein-AM loaded target tumor cells were set up in 6 replicates with 1% Triton X-100 and plain media for maximum and spontaneous release controls, respectively. The set up was incubated at 37°C in 5% CO2 for 4 hours, followed by measurement of fluorescence using BioTek Synergy 2 plate reader (Excitation: 485 nm / Emission: 530 nm). The percent specific lysis was calculated using the formula:

$$\frac{\text{(Test release} - \text{Spontaneous release)}}{\text{(Maximum release} - \text{Spontaneous release)}} \times 100$$

Global Expression Analyses

The differential expression of mRNA in NK cells was studied with Nanostring n-counter miRNA expression assay, gene expression was studied using Affymetrix 2.0 HTA gene expression array, and protein expression was assessed by reverse phase protein array (RPPA). Normal donor derived and Rosettesep purified fresh NK cells, NK cells expanded to two weeks (14 days) on K562.mbIL15 and K562.mbIL21 were used for this part of the study (Figure 5). For all samples, NK cells from 4 donors were used to attain statistical significance.

mRNA Isolation

mRNA was isolated from NK cells by RNeasy method (Qiagen, USA). Cells were collected and washed with PBS and denatured followed by ethanol phase separation. Samples were then processed on an RNeasy spin column and mRNA was eluted in ultrapure water and concentration measured using Nanodrop ND-1000 Spectrophotometer (ThermoScientific, USA); ratio of absorbance of ~ 2 was considered pure yield of mRNA and used for further analyses. For all other studies, mRNA was isolated as described after different experimental treatments of NK cells (knockdown of miRNA).

Gene Expression Analysis

Gene expression analysis was performed using Affymetrix Gene Chip HTA micro array 2.0, according to manufacturer's instructions, at the MDACC Proteomics core. The array analyzes the expression levels of over 47000 transcripts, including transcript variants of genes.

In order to identify differentially expressed genes between the groups, we analyzed the data in 2 ways. We first analyzed the differential expression of genes between fresh and expanded NK cells. This data is not used further in this study, hence not included here. We then analyzed the gene expression differences between mbIL15 and mbIL21 expanded NK cells.

Data analysis and employed statistical algorithms were carried out following the manufacturer's instructions and guidelines. The dataset analysis was performed as follows: robust multi array average (RMA) normalization was performed by background correction to eliminate noise, followed by quantile normalization in order to normalize probe intensities between the different arrays. Next we assessed the geometric density distribution of the samples by comparing the expression against density for the 4 donors, as well as the 3 groups, in order to ensure that there is 'even' distribution (Figure 7). As a final step, we applied correction for multiple testing by false discovery rate (FDR) of < 0.025. We performed two sample t-tests to identify differentially expressed genes between the two groups at the transcript level. In reporting the most significantly differentially expressed genes between the 2 groups, our parameters included mean fold difference of ≥ 2.0 fold change, and $p < 0.05$.

Volcano plot was generated using the background corrected data, and highest differentially expressed genes in both groups were assessed from this data. Heatmap was generated by euclidean hierarchical clustering and Pearson Correlation method. Individual probing of dataset was performed using the normalized data from above.

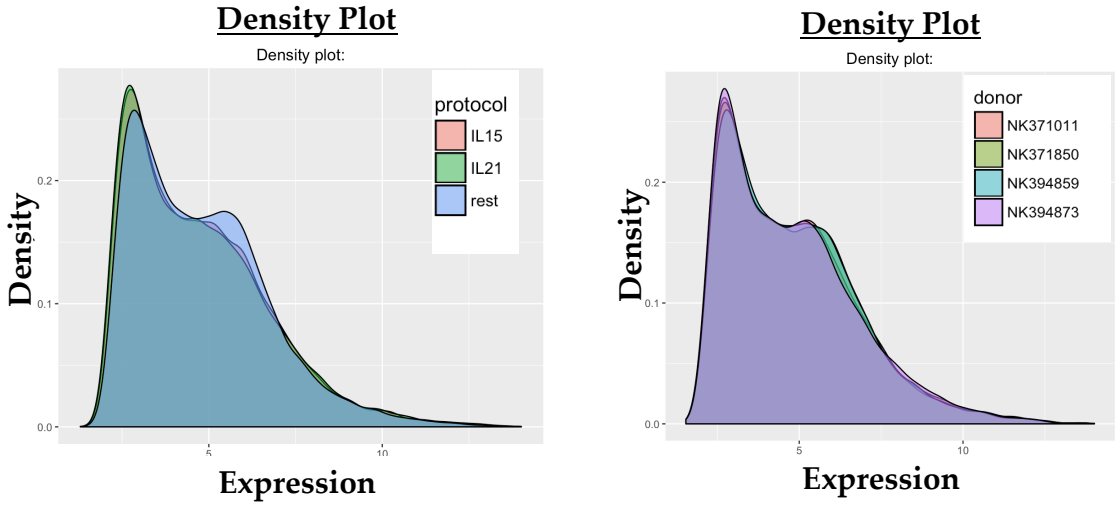


Figure 7: Verification of geometric density of mRNA Expression. Gene expression analysis was performed after data normalization and background correction. Geometric density distribution of the samples was assessed by comparison of expression against density for the 4 donors used in the study, as well as the 3 groups (fresh NK cells, mbIL15 expanded NK cells and mbIL21 expanded NK cells), an essential step to ensure that the expression analysis was performed after normalization and correction for sample loading.

Protein Isolation

As detailed for mRNA isolation, protein was isolated from the same 4 normal donor-derived fresh, mbIL15 and mbIL21 expanded NK cells based on MDACC proteomics core protocol for protein submission for RPPA analysis. Briefly, NK cells were treated with lysis buffer (1% Triton X-100, 50mM HEPES, 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 100mM NaF, 10mM sodium pyrophosphate, 1mM sodium orthovanadate and 10% glycerol), freshly added with protease and phosphatase inhibitors (Roche Applied Science). Cells were incubated on ice for 20 minutes with intermittent shaking followed by centrifugation at 14,000 rpm for 10 minutes at 4 °C. Supernatant (cell lysate) was collected and protein concentration was assessed by Pierce BCA protein assay kit (Thermofisher, USA). Cell lysate was mixed with 4X SDS sample buffer (40% Glycerol, 8% SDS, 0.25M Tris-HCL, pH 6.8, freshly supplemented with $1/10^{th}$ volume of β-mercaptoethanol), boiled for 5 minutes and submitted to the core facility to perform RPPA. Protein for western blots was isolated and prepared in a similar manner and stored in -80 °C until use.

Protein Expression Array and Analysis

Protein expression array was performed at the RPPA core as follows: 287 unique antibodies and 4 secondary antibodies as negative controls were coated on array slides. NK cell samples were run on these slides, followed by data analysis. Quality control (QC) was performed after data background correction and normalization for protein loading. A QC Score > 0.8 was considered good antibody staining, only data that scored above this range was included for further analyses. Mouse specific antibodies in the panel were eliminated from

consideration. Individual normalized values were transformed to linear values and average of the normalized linear values are represented in comparative bar graphs in the results section. To generate the heatmaps, normalized linear value was transformed to log2 values and used for hierarchical cluster analysis using Pearson Correlation and a center metric. All protein expression data from RPPA were statistically analyzed by paired student t-test method using Graphpad prism 6.0 software and $p < 0.05$ were considered significant.

miRNA Isolation

For global expression analysis, miRNA was isolated from the same 4 normal donor-derived fresh and expanded NK cells using phenol:chloroform based miRNeasy kit (Qiagen, USA). Briefly, NK cells were collected, washed with PBS and homogenized in Qiazol Lysis Reagent and chloroform, followed by centrifugation at >8000g for 15 minutes at 4°C. The upper aqueous phase containing the miRNA was carefully extracted and ethanol (molecular grade, Sigma Aldrich, USA) was added to bind RNA. The sample was then processed on an RNeasy spin column and miRNA eluted in ultrapure water. Concentration was measured using Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA) and a ratio of absorbance of ~ 2 was considered pure yield of miRNA and used for further analyses. For all other studies, miRNA was isolated as described following different experimental treatments of NK cells.

Nanostring Expression Assay

miRNA expression was analyzed by Nanostring n-Counter expression assay (143) in collaboration with Dr. Laurence Cooper's laboratory, Pediatrics, MDACC. n-Counter is a

multiplexed, direct digital detection technique that utilizes DNA-RNA hybrid capture. A total of 8 negative controls, 6 positive controls and 6 housekeeping genes are in built in to the probe set. n-Counter measures the expression of 800 miRNAs in a single step reaction without amplification by counting the actual numbers of transcripts that are captured and hybridized by a biotinylated capture probe and a fluorescently-barcoded probe juxtapositioned with each other (144). miRNA expression is represented by the number of unique sequences bound to the fluorescent probes and translated into number of counts per target. Fluorescent probe sets for n-Counter are represented as Codeset RLF File (table of reporter library file), and each fluorescent barcode corresponds to specific miRNA. The final output data for miRNA n-Counter expression is represented as Reporter Code File (RCC), and are automatically sorted based on the correct RLF file for further analysis.

NK Cell miRNA Expression Analysis

Expression data from n-Counter digital analyzer was analyzed using the accompanying n-Solver software. RCC data for miRNA expression was analyzed as follows: Quality Control (QC) was performed on raw data by background correction and subtracting negative controls from experimental counts to ensure correctness of inter-sample variability of hybridization efficiency, followed by normalization with positive controls (Figure 8). As a final step, the dataset was normalized to housekeeping genes. For this analysis, QC stringency was set at 99%. No sample normalization 'flags' was found, indicating that there were no issues with hybridization efficiency or total counts of RNA. Differential miRNA expression was analyzed in 2 ways: comparison of miRNA between primary NK cells versus expanded NK cells (both mbIL15 and mbIL21), and comparison of miRNA between mbIL15 and mbIL21 expanded NK

cells. Fold difference in expression was calculated for each sample based on the normalized counts per target values, and data was analyzed on GraphPad Prism 6 using student's t test for pair-wise comparisons.

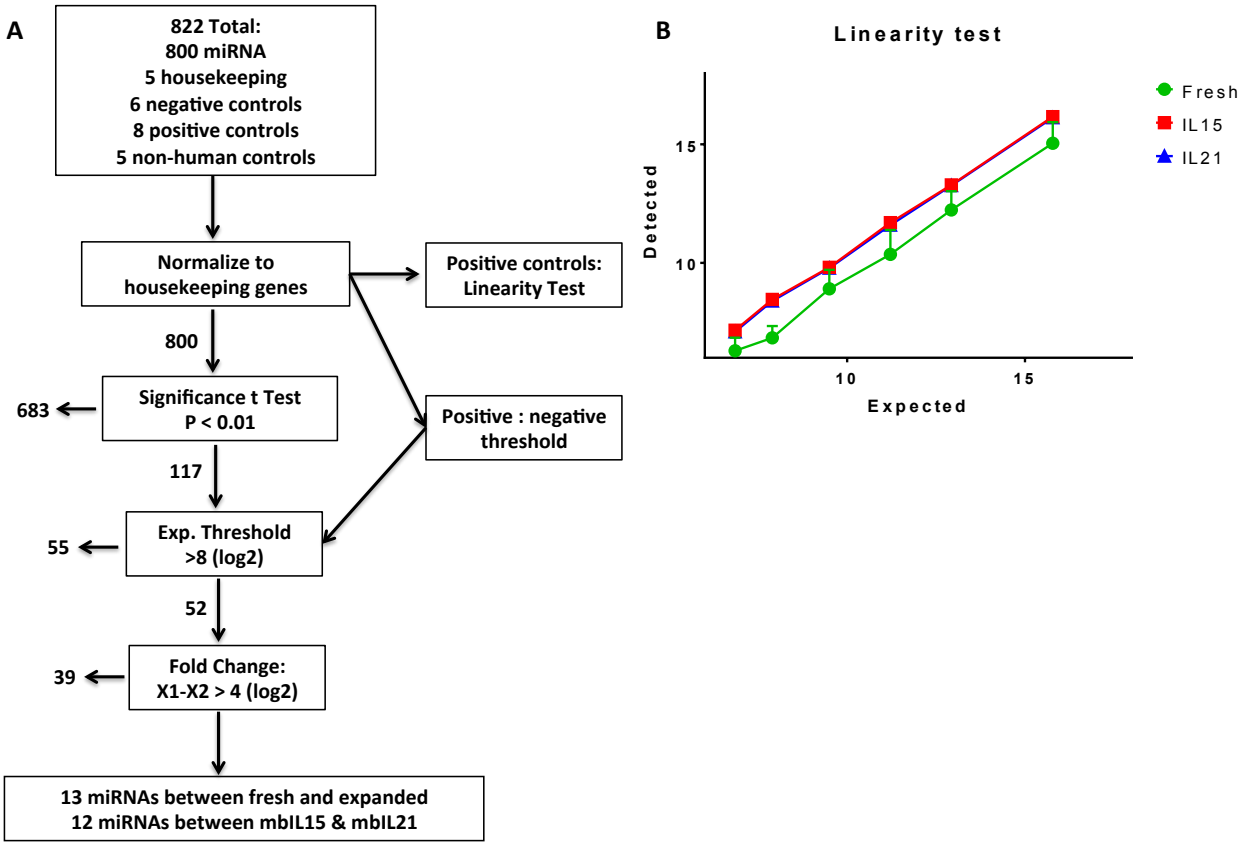


Figure 8: Schematic for miRNA data analysis. The flowchart illustrates n-Solver based miRNA analysis in NK cells. A total of 800 miRNAs were subject to normalization with positive, negative controls and housekeeping genes, followed by assessment of linearity to ensure stringent correction for sample loading and normalization. Data analysis parameters were set at high stringency as shown, with $p < 0.01$, and a threshold difference in log expression of miRNA between the groups to be > 4 (8 fold difference in expansion or more). Only miRNA that fit these criteria, and were expressed at least 8 fold or more different between mbIL15 and mbIL21 were considered for further evaluation.

miRNA Knockdown and Quantitative RT-PCR

Freshly isolated and rosette sep purified NK cells were transfected with anti-miR 124-3p, and negative controls using miRIDIAN microRNA Human hsa-miR-124-3p inhibitor and miRIDIAN microRNA Hairpin Inhibitor Negative Control (GE Healthcare Dharmacon RNA Technologies; 20 μM stock solution), respectively. NK cells were centrifuged in serum-free media at 100g for 10 minutes and resuspended in 99 μl of nucleofector solution (Nucleofector solution for human NK cells, Lonza Technologies, USA) containing 1 μl of inhibitor or negative control (final concentration: 200 nM). The nucleofection reaction was performed using Amaxa Nucleofector Device (Lonza, USA) on program X-01. The cells were maintained in serum-free media for 2 hours, followed by addition of complete media containing 2X FBS, and freshly supplemented with 50 U/ml IL-2. NK cells were collected at various time points for subsequent experimentation.

The knockdown efficiency of miR 124-3p in NK cells was measured by qRT-PCR assessment of miR 124-3p using Taqman miRNA reverse transcription kit, following manufacturer's protocol. cDNA was synthesized with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, CA) using primer/probe sets for miR-124-3p (Applied Biosystems, CA), and the data was normalized to RNU6 which was used as housekeeping control. Data were analyzed according to the comparative C_T method, and ΔΔCt values from triplicate counts was calculated and represented as comparative bar graphs. The data was further analyzed for significance using graphpad prism and $p < 0.05$ was considered significant.

CFSE Proliferation Assay

Fresh NK cells were electroporated with anti-miR 124-3p or negative control, and assessed for cellular proliferation using CellTrace CFSE Proliferation kit (ThermoFisher, USA) by flow cytometry. Live NK cells were labeled with the bright fluorescent CFSE, a DNA-binding dye that reduces its intensity with every cell cycle, and can be measured as different peaks by flow cytometry, based on the number of cell cycle occurrences in a given population. For the study here, fresh unelectroporated NK cells, NK cells electroporated with anti-miR 124-3p and negative control were loaded with 5 μM solution of CFSE dye, according to manufacturer's protocol. The CFSE loaded NK cells were then expanded on either mbIL15 or mbIL21 for 7 days. The final samples included mbIL15 and mbIL21 expansions from each of the following groups: unelectroporated NK cells, anti-miR 124-3p NK cells and negative control NK cells. Samples were collected from each of the groups after 1, 2, 3, 4 and 7 days of expansion and CFSE dilution measured by flow cytometry. The corresponding proliferation in each group was calculated based on the number of peaks, and data combined for all 4 donors, and represented as comparative bar graphs and flow cytometry histograms, to show the actual proliferative peaks. Paired student t test was performed on graphpad prism to assess significance, $p < 0.05$ was considered significant change in proliferation.

Apoptosis Assay

Similar to proliferation assay, apoptosis in NK cells following knockdown of miR 124-3p by electroporation was measured using annexin V and propidium iodide (PI) staining, and flow cytometry. Unelectroporated NK cells were expanded on mbIL15 and mbIL21 for 7 days,

and stained with AnnexinV and PI, to evaluate baseline rate of apoptosis induced due to IL-15 and IL-21 signaling. Fresh NK cells from the same donors were also electroporated with either anti-miR 124-3p or negative control, and apoptosis measured in these cells in a similar manner. Percentage apoptotic, live and dead cells form the 3 donor NK cells tested were combined, student t test was performed using graphpad prism, and data represented as comparative bar graphs as well as flow plots.

Gene Expression by qPCR

The knockdown effect of miR 124-3p on gene expression in NK cells was assessed by quantitative RT-PCR. Fresh NK cells were electroporated with anti-miR 124-3p and negative control; unelectroporated cells were used as baseline. mRNA was isolated from unelectroporated, miR 124-3 knockdown and negative control NK cells at time points 1, 2, 3 day, by the method described above. Gene expression was analyzed by the 2 step RT-PCR reaction using SYBR Green RT-PCR Master Mix (Applied Biosystems, USA) as follows: first, cDNA was prepared from 500 ng of mRNA as template from all the samples using a mixture of random hexamers, deoxy NTPs and reverse transcriptase enzyme in a thermal cycler, according to manufacturer's protocol. Next, PCR was performed using the forward and reverse primers / probe set (forward and reverse primer sets are given below), and SYBR Green PCR Master Mix, and the reaction was performed on Roche LightCycler 480, according to manufacturer's protocol. The data was normalized to GAPDH expression, and analyzed by comparative C_T method, $\Delta\Delta$Ct values from triplicate counts was calculated and represented as comparative bar graphs.

To measure the effect of IL-15 and IL-21 signaling, NK cells were electroporated with miR 124-3p, negative controls and unelectroporated cells followed by expansion with mbIL15 or mbIL21 for 7 days, and $CD56^{+}NKp46^{+}$ NK cells were sorted by Fluorescence Activated Cell Sorting (FACS) at the MDACC flow core. mRNA was isolated and qRT-PCR was performed on these samples as before.

Primer sets used:

BCL2L11: FOR 5' TGGCAAAGCAACCTTCTGATG 3',

REV 5' GCAGGCTGCAATTGTCTACCT 3'

STAT 3: FOR 5' ATCACGCCTTCACAGACTGC 3';

REV 5' CATCCTGGAGATTCTCTACCACT 3'

HTERT: FOR 5' CGGAAGAGTGTCTGGAGCAA 3';

REV 5' TGACCTCCGTGAGCCTGTC 3'

GAPDH: FOR 5' CGTGAGGTCCGTTAGGAAAA 3';

REV 5' ATAGTGGGATGCGAGTCCAG 3'

Western Blot

5 normal donor-derived and rosette sep purified NK cells were electroporated with anti-miR 124-3p and negative controls as before; unelectroporated samples were used as baseline. Protein was isolated and quantitated from all these samples by the method described previously. Western blot analysis was performed for assessment of Stat 3, phospho Stat3, TERT and Bim, using monoclonal antibodies against anti-STAT3, anti-STAT 3 phospho

Y705, anti-STAT3 phospho Y727, anti-telomerase reverse transcriptase (Abcam, USA) and anti-Bim (Cell Signaling Technologies, USA). The protein expression was normalized to β-actin, used as housekeeping control. Antibody-reactive proteins were detected with horseradish peroxidase–labeled specific secondary antibodies, and the membrane was developed by chemiluminescence (ECL; Amersham) on Kodak Image station. Protein levels were measured and quantified using ImageJ Version 1.43 software.

CHAPTER 4: RESULTS AND DISCUSSION

mbIL15 And mbIL21 Lead To Differential Expansion Of NK Cells

We have previously extensively compared the attributes of NK cells expanded on K562.mbIL15 and K562.mbIL21 (mbIL15 and mbIL21 respectively), since both expansion platforms are based on feeder cells modified to express membrane bound cytokines (Table 1). We assessed the cytotoxicity abilities of NK cells expanded for 14 days on either mbIL15 or mbIL21 from the same donors by calcein release assay, and reported that NK cells expanded on mbIL21 was marginally better than mbIL15 expanded NK cells against K562, a target cell line used as a standard of cytotoxicity measure of NK cells (62).

In the current study however, when we tested expanded NK cells against other targets such as AML, we observed that mbIL21 expanded NK cells was significantly better in cytotoxicity (Figure 9A & 9B). Next, when we assessed the phenotype and receptor expression between mbIL15 and mbIL21 expanded NK cells on the 4 donors used in this study, we observed that most of the receptors evaluated were similar in both the percentage and relative expression (MFI), with very few differences (Figure 10A). We observed the relative expression of CD11a (LFA-1), an adhesion molecule and NKp46, the activating receptor to be higher in mbIL21 expanded NK cells, whereas the relative expressions of CD27, a maturation marker and NKg2A, an inhibitory receptor were higher in mbIL15 expanded NK cells (Figure 10B). CD11a/ LFA-1 is essential for strong adhesion and target cell lysis by NK cells, deficiency in LFA-1 has been associated with impaired NK cell cytotoxicity. The role of LFA-1 in NK cell cytotoxicity is particularly essential against leukemia (145). NKp46 is an NCR

and is a key activating receptor involved in improved NK cell cytotoxicity. A decrease in CD27 indicates the maturation status of NK cells, and increased granular content, thereby increased effector cell phenotype (146).

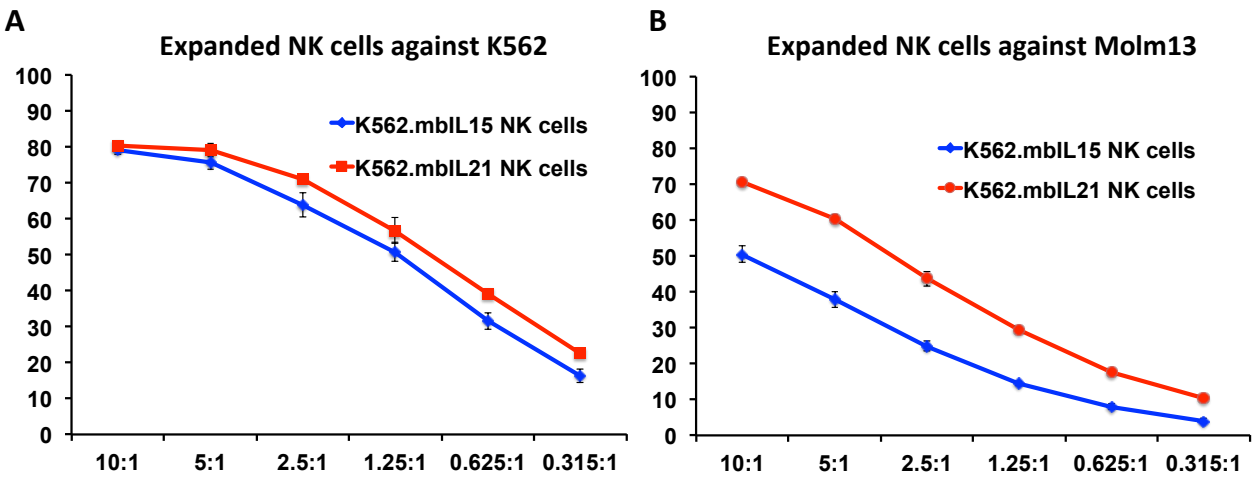
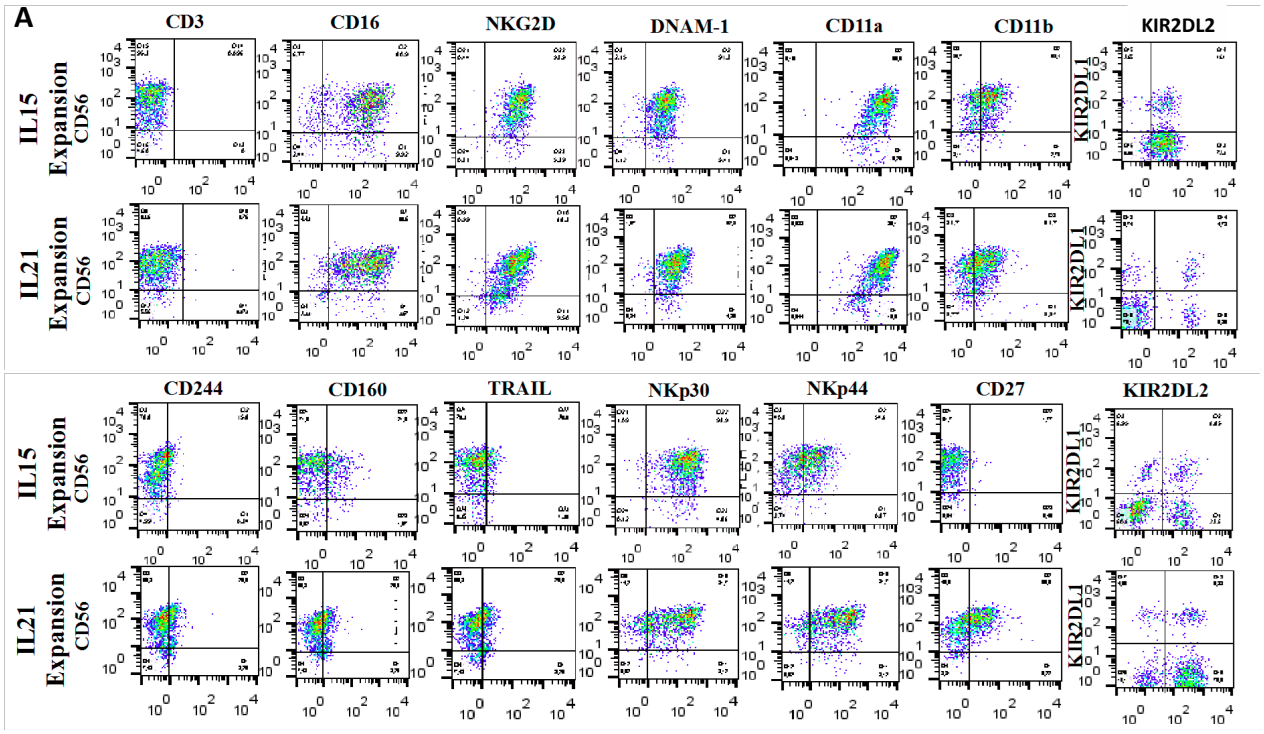


Figure 9: mbIL21 expanded NK cells have higher cytotoxicity potential. (A) mbIL15 and mbIL21 expanded NK cells from the same donor were analyzed for cytotoxicity against K562. As shown, NK cells expanded by both methods yielded high cytotoxic ability, with mbIL21 expanded cells performing better in a non-significant trend. (B) Assessment of cytotoxicity against Molm 13, an AML cell line indicates that mbIL21 has significantly higher cytotoxicity compared to mbIL15 expanded NK cells at all E:T ratios ($p < 0.05$). The 4 NK donors used for the global differential expression analysis were used for this study; data shown is representative of 4 donors tested.


A
CD3
CD16
NKG2D
DNAM-1
CD11a
CD11b
KIR2DL2
IL15 Expansion CD56
IL21 Expansion CD56
KIR2DL1
CD244
CD160
TRAIL
NKp30
NKp44
CD27
KIR2DL2
IL15 Expansion CD56
IL21 Expansion CD56
KIR2DL1

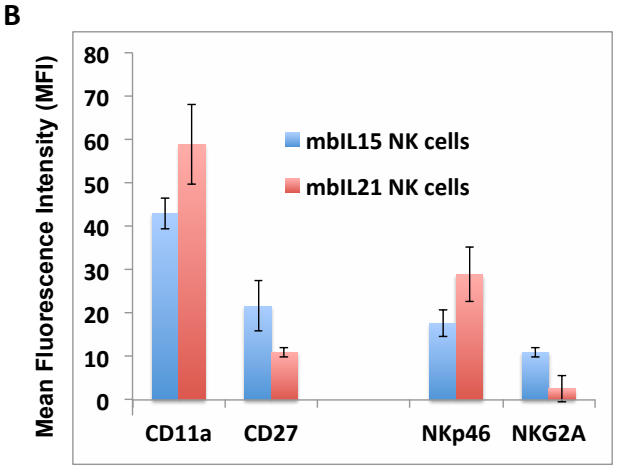
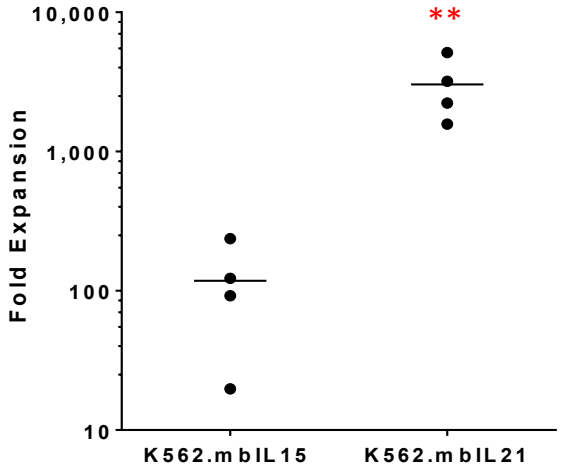

B
Mean Fluorescence Intensity (MFI)
80
70
60
50
40
30
20
10
0
mbIL15 NK cells
mbIL21 NK cells
CD11a
CD27
NKp46
NKG2A

Figure 10: NK cells expanded on mbIL15 and mbIL21 are phenotypically similar. (A) Flow cytometry analysis of activating and inhibitory receptors following mbIL15 and mbIL21 from the same donor shows similarity in the expression of most of the receptors. Only a few of the receptors showed differences in their expression levels. Data shown is representative dot plots for one of the 4 donors tested. Significance was assessed by one-way anova. (B) Only the phenotypic markers and receptors that showed differential expression (CD11a, CD27, NKp46 and NKG2A) between mbIL15 and mbIL21 are represented. Data shown is an average of expression levels from the 4 donors tested. One way anova was performed on prism to assess the significance in differential expression.

The largest and the most significant observable difference between mbIL15 and mbIL21 expanded NK cells were in the fold expansion, where mbIL21 outperforms mbIL15 expansion by several thousand folds consistently (Figure 2). Similar to previously published data, we observed an increased expansion of NK cells from all 4 donors on mbIL21 after 2 weeks (Figure 11). As discussed previously, this difference in fold expansion of NK cells may be attributed to factors including maintenance of telomere length in mbIL21 NK cells, and STAT3 mediated signaling in mbIL21, as opposed to STAT5 mediated signaling in mbIL15, but there may be other factors involved in causing this effect. There are not enough evidences in literature to explain the tremendous difference in expansion due to IL-15 and IL-21 signaling. Hence we focused our efforts on delineating the differentially expressed genes and their specific methods of gene regulation.

A



B

	Fold Expansion	
	K562.mbIL15	K562.mbIL21
Donor 1	123	2226
Donor 2	19.75	3193
Donor 3	92	1575
Donor 4	237	5133

Figure 11: mbIL21 expansion is more robust and superior compared to mbIL15. The 4 normal donor derived NK cells used for the global profiling of gene, protein and miRNA expression were assessed for their rate of expansion on mbIL5 and mbIL21 for 2 weeks. The mbIL21 expansion method was consistently more prolific and higher numbers of activated NK cells. Statistical analysis for differential expansion was performed using non-parametric student t test on Graphpad prism. This difference in expansion potential between mbIL15 and mbIL21 expansions, and the robustness of IL-21 signaling in causing this expansion are the premise of the study conducted here.

NK Expansion On mbIL15 And mbIL21 Differentially Regulate Gene And Protein Expression

NK cells expanded on mbIL15 and mbIL21 differ in expression of hTERT, due to which NK cells undergo replicative senescence (70), and expansion with mbIL21 significantly increased telomere length compared to mbIL15 expansion (62), as discussed previously. The role of telomeres and telomerase reverse transcriptase (hTERT) are well established in the context of cellular longevity, replication and senescence. Telomeres are physical end of chromosomes that shorten with each replicative cycle. To prevent this, the ribonucleoprotein enzyme telomerase along with its catalytic subunit hTERT adds TTAGGG repeats to the end of chromosomes (147-149). Aberrant overexpression of hTERT is linked to immortalization of cells, enhancement of tumor cell proliferation, as well as increased invasiveness and metastatic potential (147, 150-152). However these changes caused due to the increase of hTERT in mbIL21 expansion is beneficial to NK cells in that cellular proliferation is increased several fold leading to an overall improved expansion as shown, unlike any other platform before. This observation concurs with previous studies in normal human cells, where overexpression of telomerase stabilized telomeres and extended their replicative life span by at least 20 doublings (153). Conversely, loss of hTERT has been shown not only to induce telomere loss, but non-prevention of stress induced senescence as well as apoptosis (154). Senescence in NK cells expanded on mbIL15 has been reported previously, however apoptosis has not been linked to mbIL15 expansion thus far. Further, the role of IL21 in (potentially preventing) apoptosis has not been shown in literature, and could shed more insight into the possible repertoire of cellular mechanisms that happen synchronously in order to cause the observed expansion outcome.

As detailed in the introduction, STATs are critical transcription factors that regulate IL-15 and IL-21 mediated signaling. IL-21 signals predominantly through STAT3 via activation of JAK 1, and IL-15 signals predominantly through STAT5 via activation of JAK 3. The traditional role of STAT5 and STAT3 in NK cells is to promote survival, maturation and proliferation and activation of NK cells (155), however, the extent to which these functions are exerted differs greatly between the 2 transcription factors. In the context of activation and proliferation of NK cells in vivo, STAT5 acts as a master regulator (156), however depending on other cytokine signals STAT5 regulation could act to convert NK cells from cytotoxic killers to tumor promoters (156). We recently reported that STAT3 plays a critical role in NK cell proliferation, NKG2D expression, and cytotoxicity (157). It is well established that dominant negative STAT3 mutations lead to NK cell deficiencies such as Job's Syndrome or hyper-IgE syndrome, characterized by recurrent bacterial skin and lung infections (158-161).

Given the role of Stats and hTERT in the expansion outcome of NK cells, we sought to further understand the role of additional proteins and pathways that could mediate the observed differences in expansion between mbIL15 and mbIL21. We focused our efforts on understanding additional molecular mechanisms by assessing the global gene expression profiles using Affymetrix Human Transcriptome HTA micro array 2.0 and protein expression by RPPA.

Gene Expression

We assessed the differential gene expression between mbIL15 and mbIL21 expanded NK cells with 4 samples each. Additionally, we also assessed the differential expression of

genes between fresh and expanded NK cells. This data is not used further in this study, but future research utilizing this data is under consideration.

Upon interrogation of gene expression of over 47,000 transcripts, we found that there were only a handful of genes that seemed to be differentially expressed. However this seems sufficient to drive a significant difference in the observed expansion between the 2 groups. We interrogated the gene expression data by looking for the most highly expressed genes in the mbIL21 cohort, and conversely genes that were lowest expressed in mbIL21 but very high in mbIL15 cohort which are represented in the far extreme spectrum of our analysis (Figure 12). A comparison of the highest scoring genes between fresh NK cells, as well as NK cells expanded on mbIL15 and mbIL21 is represented in Figure 13, where the heatmap indicates the expression levels of our highest scoring genes. Further, we correlated our findings with their known functions in NK cells and other lymphocytes (Table 3).

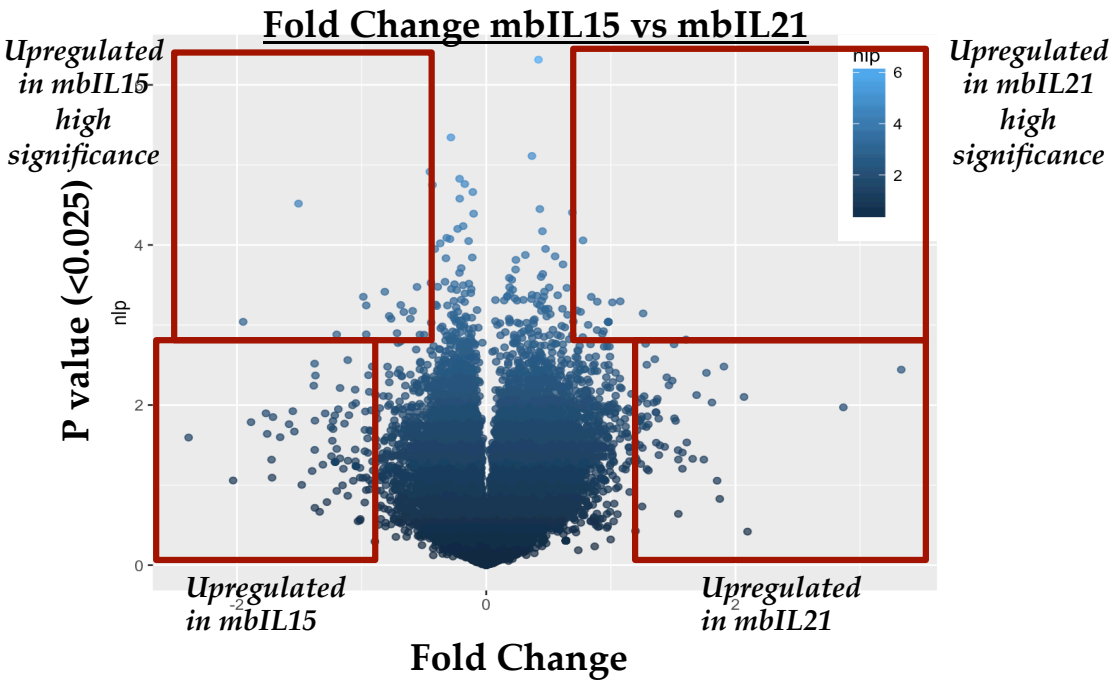


Figure 12: Volcano plot of mRNA gene expression analysis. NK cells expanded with mbIL15 and mbIl21 were assessed for differential gene expression by microarray. Data indicates only a few genes are differentially expressed between the 2 groups. Volcano plot indicates fold change of expression plotted against significance. Data analysis was performed by specifically interrogating highest expressing genes in mbIL21, thus the left side indicates high expression, and the right extreme negative to lower expression for mbIL21 expanded cells. Conversely, right extreme indicates high expression and left extreme low expression for mbIL15 NK cells. Genes on the very top are high significance, with higher expression in both cohorts. A list of genes that are differentially expressed between the groups was derived from this data.

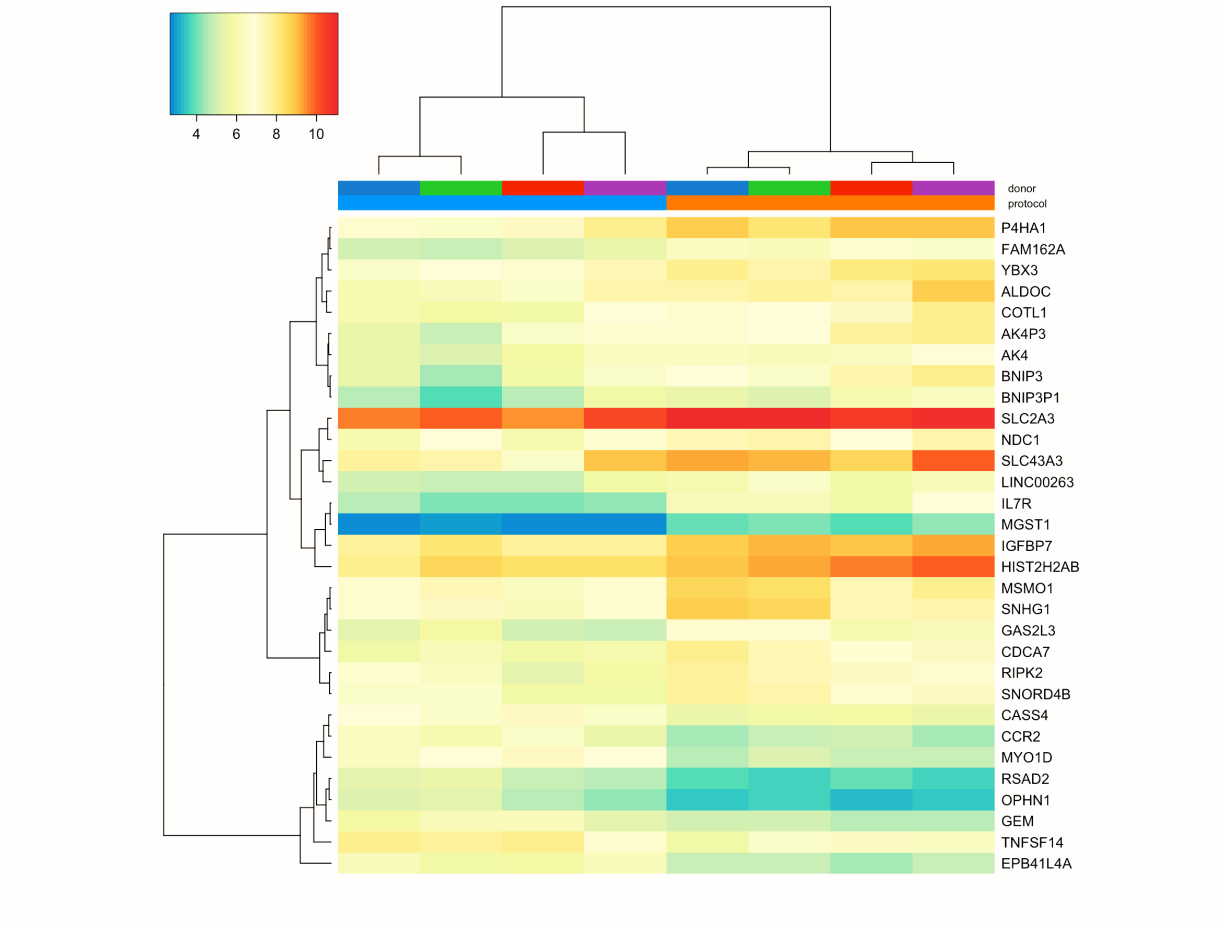


Figure 13: Differential gene expression between mbIL15 and mbIL21 expanded NK cells. Highest expressing genes in both expansion methods were first assessed by comparison of fold change and significance ($p < 0.025$). The differentially expressed gene set was derived from this large dataset, where both high and low expressing genes in both set of samples were taken into consideration. Heatmap indicates a panel of the most significantly differentially expressed geneset between expansion cohorts. Comparisons between fresh and expanded cells were also assessed, but are not included here. Future studies evaluating fresh versus expanded cells are under consideration.

A

Gene upregulated in mbIL15 Expanded NK cells	Function
RSAD2	Activates NFkB activation
BCL2L11	Pro-apoptotic protein, induces apoptosis
ACVR2A	TGFb superfamily protein
CCR2	Essential for viral infection mediation
EPB41L4A	Cytoskeletal rearrangenent
ITGA1	Subunit of LFA1, an adhesion protein
MYO6	Upregulated in adaptive NK cells
CD44	Lymphocyte activation
CD70	Activation and cytotoxic function of NK cells
TNFSF14	Stimulation/ proliferation of T cells; apoptosis induction in tumor cells
CEACAM1	Mediates suppression of NKG2D mediated NK cell cytotoxicity
TNFSF4	OX40L
LINC00599	LINC RNA
GEM	GTP binding reg protein, signal transduction; Ras GTPases

Table 3A: Functional correlation of highly expressed genes in mbIL15 expanded NK cells. Several genes related to enhancing cytotoxicity (indicated with blue arrows) and cellular proliferation (indicated with green arrow) was differentially highly expressed in mbIL15 and mbIL21 expanded NK cells. The mbIL15 expanded NK cells had high expression of cytotoxicity related genes. Of the genes that were related to proliferation and apoptosis, BCL2L11, the gene encoding pro-apoptotic protein Bim was highly expressed, indicating a survival disadvantage to NK cells.

B

Gene upregulated in mbIL21 Expanded NK cells	Function
AK4	Activates cell cycle progression
NDC1	Acts as an MTOC anchor
MIR15B	Activates BCL2, an anti-apoptotic protein
IGFBP7	Regulates apoptosis, cell growth and angiogenesis
HIST2H2AB	Promotes Cellular proliferation
IL7R	Blocks apoptosis in T cells
MGST1	Prevents oxidative stress accumulation in cells
GPI	Glycolytic enzyme promotes glucose metabolism
SDK2	Promotes synaptic connectivity
DTL	E3 ubiquitin-protein ligase complex required for cell cycle control, promotes cellular proliferation
RIPK2	Potent NFkB activator in immune cells
KLF10	Transcriptional repressor of TGFb signaling
BNIP3	Interacts with BCL2, an anti-apoptotic protein
ALDOC	protein in glycolytic pathway, increases glycolysis

Table 3B: Functional correlation of highly expressed genes in mbIL21 expanded NK cells. Several genes related to enhancing cytotoxicity (indicated with blue arrows) and cellular proliferation (indicated with green arrow) was differentially highly expressed in mbIL15 and mbIL21 expanded NK cells. Expansion of NK cells with mbIL21 had an opposite effect on the expression of genes. Genes related to increasing cellular proliferation, cell cycle and glycoysis,

prevention of oxidative stress and apoptosis were higher, along with genes related to increasing cytotoxicity.

Amongst the genes highly expressed in mbIL15 expanded NK cells, several of the genes are associated with enhancing cytotoxicity functions in lymphocytes. These genes include RSAD2, CCR2, ITGA1, CD44 and CD70. There were some genes that are contrasting, such as CEACAM1, TNFSF4 and ACVR2A. In the context of expansion of NK cells, there were far fewer genes that showed significant differences. It was however interesting to note that the gene expression profile correlates well with the observed differential expansion. For instance, mbIL15 expanded NK cells showed an overall decreased expression of proliferative markers, and an increased expression of apoptotic markers such as TNFSF14 and BCL2L11. Additionally, previously uncharacterized genes such as KIAA0825 and LINVC00599 also flagged as very highly expressed within the mbIL15 cohort (Table 3A).

In the mbIL21 expanded NK cells, several more genes were upregulated and the gene expression pattern was very different; we observed a reverse trend to that of mbIL15 NK cells. Of the most highly expressed genes, there were some that were related to increasing NK cell cytotoxicity such as RIPK2, KLF10, NDC1, CD109 and SDK2. No genes involved in diminishing cytotoxicity potential were in this group. Several of the genes involved in enhancing functions such as cellular proliferation, metabolism, cell cycle progression, prevention of apoptosis and oxidative stress were highly expressed. These genes include HIST2H2AB, ENO1, AK4, MIR15B, GP1, MGST1, DTL, BNIP3, ALDOC and DPP4 (Table 3B). A detailed list of the most highly expressed genes and their related functions is included in Table 4. It is interesting to note that while IL-21 signaling in vivo is linked more to cytotoxicity function rather than proliferation, mbIL21mediated activation and signaling has

increased expression of a large cohort of genes that helps promote cell cycle progression, proliferation, and prevent apoptosis, thereby enhancing the overall prolific expansion.

Of particular interest, genes that are involved in enhanced glucose metabolism and reduction of oxidative stress such as MGST1, GP1, DT1 and ALDOC were significantly upregulated in mbIL21 expanded NK cells. Considering the active proliferation and cell cycle ongoing due to IL-21 mediated expansion, NK cells would be subject to a rapid build up of oxidative stress due to increased uptake of glucose and higher metabolic activity, resulting in an increase in redox state in cells. Higher expression of genes that are involved in reducing oxidative stress in NK cells indicates that the robust activation of glucose metabolism due to higher cellular turnover and the resultant redox increase would be nullified by these enzymes, thereby promoting favorable cellular environment for unimpeded growth potential, as observe with mbIL21 expansion.

Genes upregulated in mbIL15	Functional Group
EPB41L4A, MYO6, TNFSF8,	Cellular growth
BCL2L11	Growth inhibition
TNFSF4, RSAD2, CR2, ITGA, CD44, CD70, TNFSF14	Cytotoxicity
CEACAM1, ACVR2A	Cytotoxicity inhibition
GEM, PLXDC2, PLEKHA1, ARHGEF12, MYO1D, CASS4	Cellular Signaling
LINC00599, DYNC2H1	Unknown function
Genes upregulated in mbIL21	**Functional Group**
DTL, HIST2H2AB, AK4, CDCA7, DPP4, FAM162A, MIR15B, GBE1, MSMO1, IGFBP7, SLC7A11, IL7R MYB, CDCA7L, RIPK2, KLF10, BNIP3, MGST1, ALDOC, GP1	Cellular growth
-	Growth inhibition
CD160, NDC1, CD109, HLA-DRA KLF10, CD68	Cytotoxicity
-	Cytotoxicity inhibition
PGK1, SCD, PDLIM3, DHCR7, NUCB2, SNHG1, GAS2L3, AK4P3,	Cellular Signaling
LINC00263	Unknown function

Table 4: Most highly expressed genes in mbIL15 and mbIL21 expanded NK cells and their broad cellular functions.

Further, given the previously established roles of STATs and TERT in mediating proliferation and senescence, respectively, and that BCL2L11 (gene encoding pro-apoptotic protein Bim) was highly expressed in mbIL15 expanded NK cells, we specifically interrogated the gene expression of Stat 3, Stat 5, TERT and BCL2L11 in expanded NK cells. We found that upon expansion, expression of Stat 3 and Stat 5 were similar in both mbIL15 and mbIL21. Similarly, TERT yielded no significant difference in expression, with mbIL21 cells showing marginally higher expression (Figure 14). It is possible that continual activation and expression of Stats and TERT for 2 weeks during expansion may have normalized the expression levels, and it is also possible that the differences may be greater during an earlier time during expansion, such as 24 to 48 hours. However, due to the presence of contaminating feeder cells in the culture early in the expansion phase, it was not experimentally feasible to perform analysis. It is however, interesting to note that despite no significant difference in expression levels, Stats and TERT are critical to the expansion outcome.

The largest and the most significant difference between mbIL15 and mbIL21 were observed in the expression of both transcripts of BCL2L11, with high expression in mbIL15 NK cells (Figure 14). BCL2L11 belongs to the BCL-2 family of pro-apoptotic genes that promote cellular death by cytochrome C release upon death signal activation in several cell types, including immune cells (162). BCL2L11 expression was also found to be high in fresh NK cells, consistent with previous findings, the high expression of BCL2L11 in peripheral blood NK cells, and subsequent high Bim expression are essential for the maintenance of leukocyte homeostasis and preclude autoimmunity under normal conditions in vivo(163). Additionally, this corresponds with our findings that mbIL15 expansion increased NK cell susceptibility to apoptosis, as evidenced by increase in Annexin V positive apoptotic cell

population only after 7 days (Figure 15). This finding indicates that BCL2L11, and corresponding Bim mediated apoptosis in mbIL15 is an active and ongoing process throughout the expansion cycle in vitro, lending a certain disadvantage to the overall expansion of NK cells using mbIL15.

The changes observed with the expression of BCL2L11 and other apoptotic, cellular proliferation and cell cycle genes prompted us to evaluate their protein expression profiling in order to confirm whether the findings with gene expression data could be correlated in the protein expression. Hence we performed RPPA for assessment of differentially expressed proteins in NK cells.

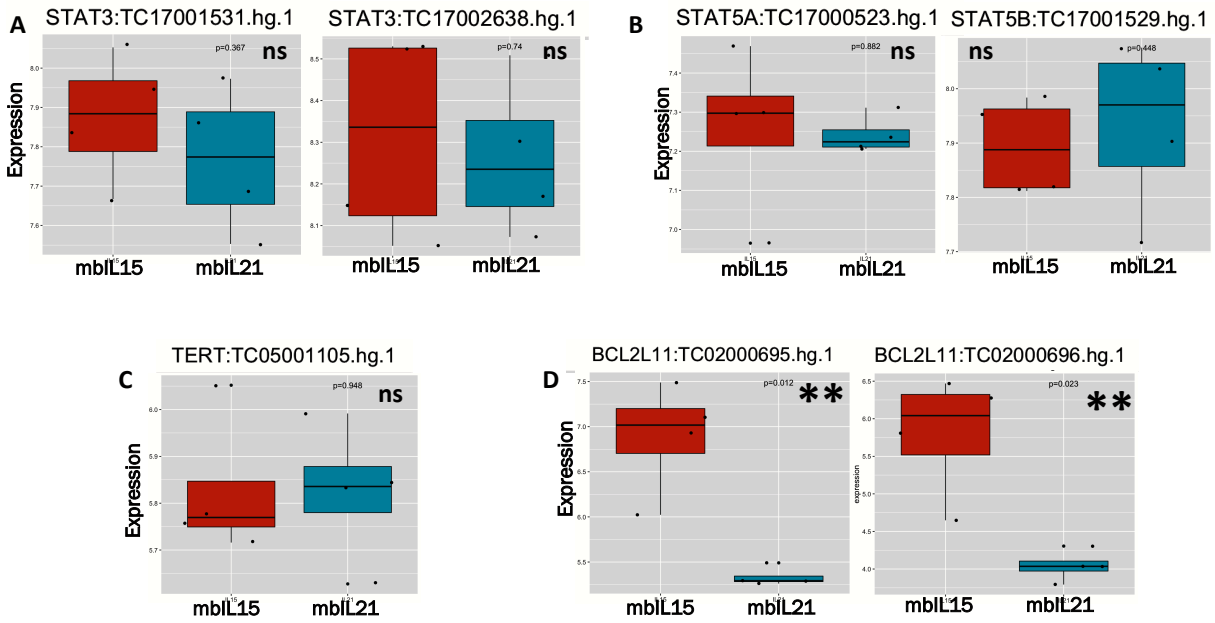


Figure 14: Interrogation of differential expressions of specific genes between mbIL15 and mbIL21. Expression levels of Stat 3, Stat 5, TERT (due to their previously established roles in causing the differential expansions) and BCL2L11 were specifically interrogated. Stats and TERT ($p > 0.05$) did not show any difference in their expression levels, although TERT showed a marginal difference with higher expression in mbIL21 NK cells. BCL2L11 was significantly different between the 2 groups ($p < 0.01$) very high expression in mbIL15 and significantly lower in mbIL21 NK cells, indicating a clear survival advantage in mbIL21 NK cells. We focused on this finding for further evaluation, specifically post-transcriptional regulation of BCL2L11, as well as Stats and TERT during early phase of expansion.

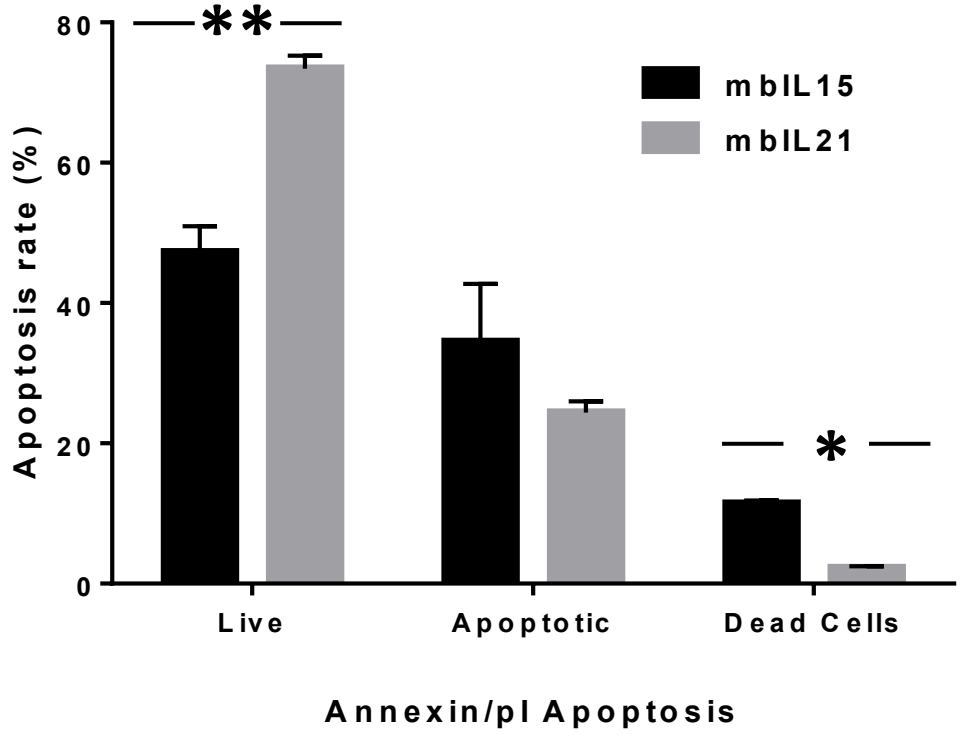
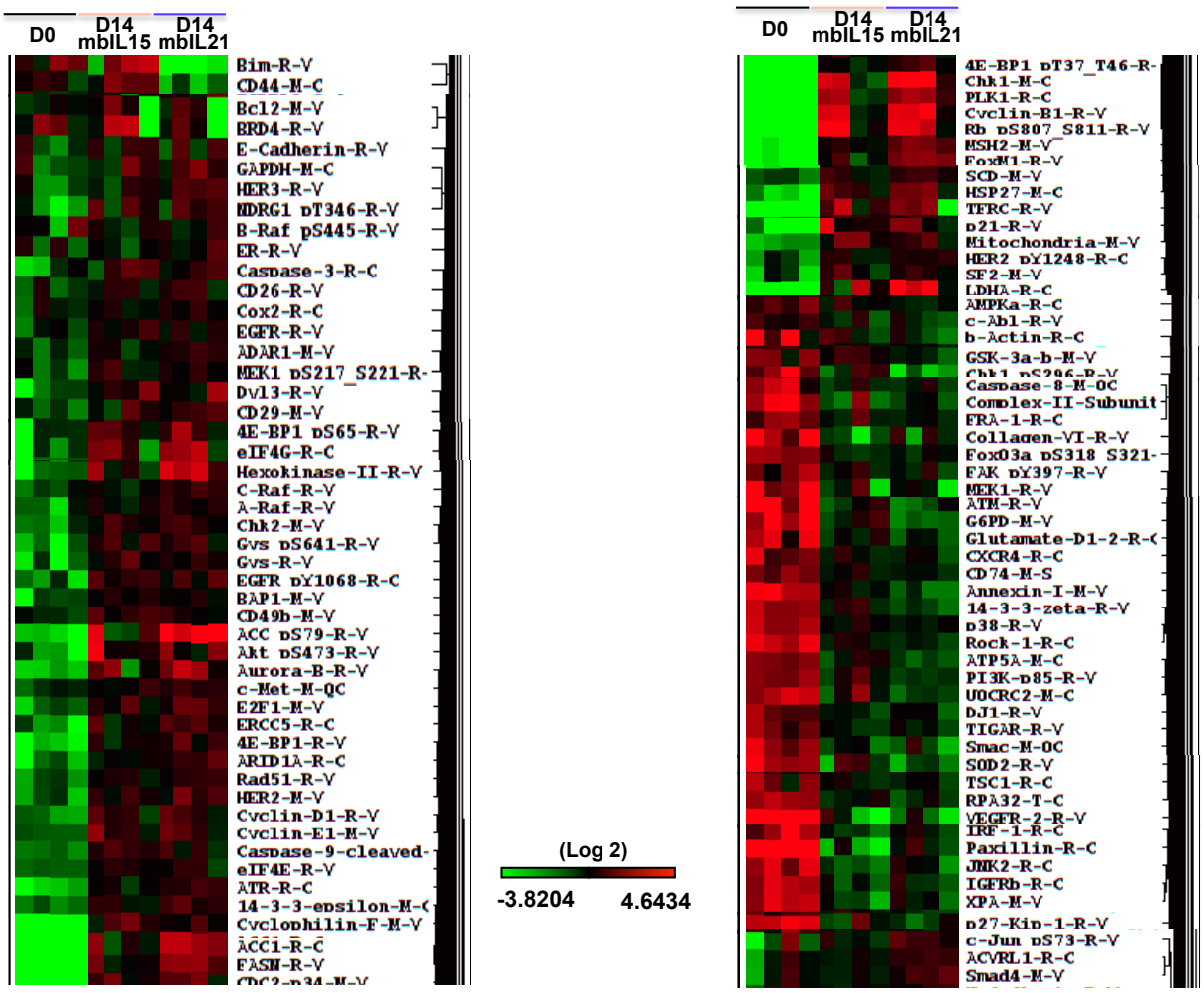


Figure 15: Comparison of apoptosis between mbIL15 and mbIL21 NK cells. NK cells from 3 normal donor-derived buffy coats were expanded on either mbIL15 or mbIL21. At the end of stim 1 (7 days), CD56+NKp46+ NK cells were assessed for apoptosis by Annexin V staining. As shown, mbIL15 expansion increased the rate of apoptosis and decreased live cells, whereas mbIL21 expansion indicated higher percentage live cells ($p < 0.01$) and lower percentages of apoptotic and dead cells ($p < 0.05$). The changes in apoptosis due to IL-15 and IL-21 signing and a possible caveat to continual stimulation with IL-15 as opposed to IL-21 has not been previously reported, and is a new finding from this study.

RPPA Protein Expression

The differential protein expression was assessed in a similar manner to gene expression comparing expression of fresh and expanded NK cells, as well as comparison between mbIL15 and mbIL21 NK cells. The RPPA panel consisted of 280 proteins, and included several genes that are critical for cell cycle, proliferation, apoptosis and metabolism. In our comparative analysis of protein expression, we scored the proteins in terms of their highest expression, followed by the largest differential expression between mbIL15 and mbIL21 cohorts. The overall protein expression is represented as a heat map generated by supervised euclidean hierarchical clustering; the data represents a snap shot of the initial screening and represents the overall differences between fresh and expanded NK cells (Figure 16A). As can be observed from the expression pattern, several proteins are differentially expressed between day 0 fresh NK cells and expanded NK cells, However, as was the pattern with gene expression, only a few proteins were differentially expressed between mbIL15 and mbIL21 NK cells. The highest scoring proteins amongst the expanded cells were determined from this data and further evaluated for their differential expression between mbIL15 and mbIL21 (Figure 16B).

A


D0
D14 mbIL15
D14 mbIL21
Bim-R-V
CD44-M-C
Bcl2-M-V
BRD4-R-V
E-Cadherin-R-V
GAPDH-M-C
HER3-R-V
NDRG1 pT346-R-V
B-Raf pS445-R-V
ER-R-V
Caspase-3-R-C
CD26-R-V
Cox2-R-C
EGFR-R-V
ADAR1-M-V
MEK1 pS217_S221-R-
Dvl3-R-V
CD29-M-V
4E-BP1 pS65-R-V
eIF4G-R-C
Hexokinase-II-R-V
C-Raf-R-V
A-Raf-R-V
Chk2-M-V
Gys pS641-R-V
Gys-R-V
EGFR pY1068-R-C
BAP1-M-V
CD49b-M-V
ACC pS79-R-V
Akt pS473-R-V
Aurora-B-R-V
c-Met-M-QC
E2F1-M-V
ERCC5-R-C
4E-BP1-R-V
ARID1A-R-C
Rad51-R-V
HER2-M-V
Cyclin-D1-R-V
Cyclin-E1-M-V
Caspase-9-cleaved-
eIF4E-R-V
ATR-R-C
14-3-3-epsilon-M-(
Cyclophilin-F-M-V
ACC1-R-C
FASN-R-V
(Log 2)
-3.8204
4.6434
D0
D14 mbIL15
D14 mbIL21
4E-BP1 pT37_T46-R-
Chk1-M-C
PLK1-R-C
Cyclin-B1-R-V
Rb pS807_S811-R-V
MSH2-M-V
FoxM1-R-V
SCD-M-V
HSP27-M-C
TFRC-R-V
p21-R-V
Mitochondria-M-V
HER2 pY1248-R-C
SF2-M-V
LDHA-R-C
AMPKa-R-C
c-Abl-R-V
b-Actin-R-C
GSK-3a-b-M-V
Caspase-8-M-QC
Complex-II-Subunit
FRA-1-R-C
Collagen-VI-R-V
FoxO3a pS318 S321-
FAK pY397-R-V
MEK1-R-V
ATM-R-V
G6PD-M-V
Glutamate-D1-2-R-(
CXCR4-R-C
CD74-M-S
Annexin-I-M-V
14-3-3-zeta-R-V
p38-R-V
Rock-1-R-C
ATP5A-M-C
PI3K-p85-R-V
UQCRC2-M-C
DJ1-R-V
TIGAR-R-V
Smac-M-QC
SOD2-R-V
TSC1-R-C
RPA32-T-C
VEGFR-2-R-V
IRF-1-R-C
Paxillin-R-C
JNK2-R-C
IGFRb-R-C
XPA-M-V
p27-Kip-1-R-V
c-Jun pS73-R-V
ACVRL1-R-C
Smad4-M-V

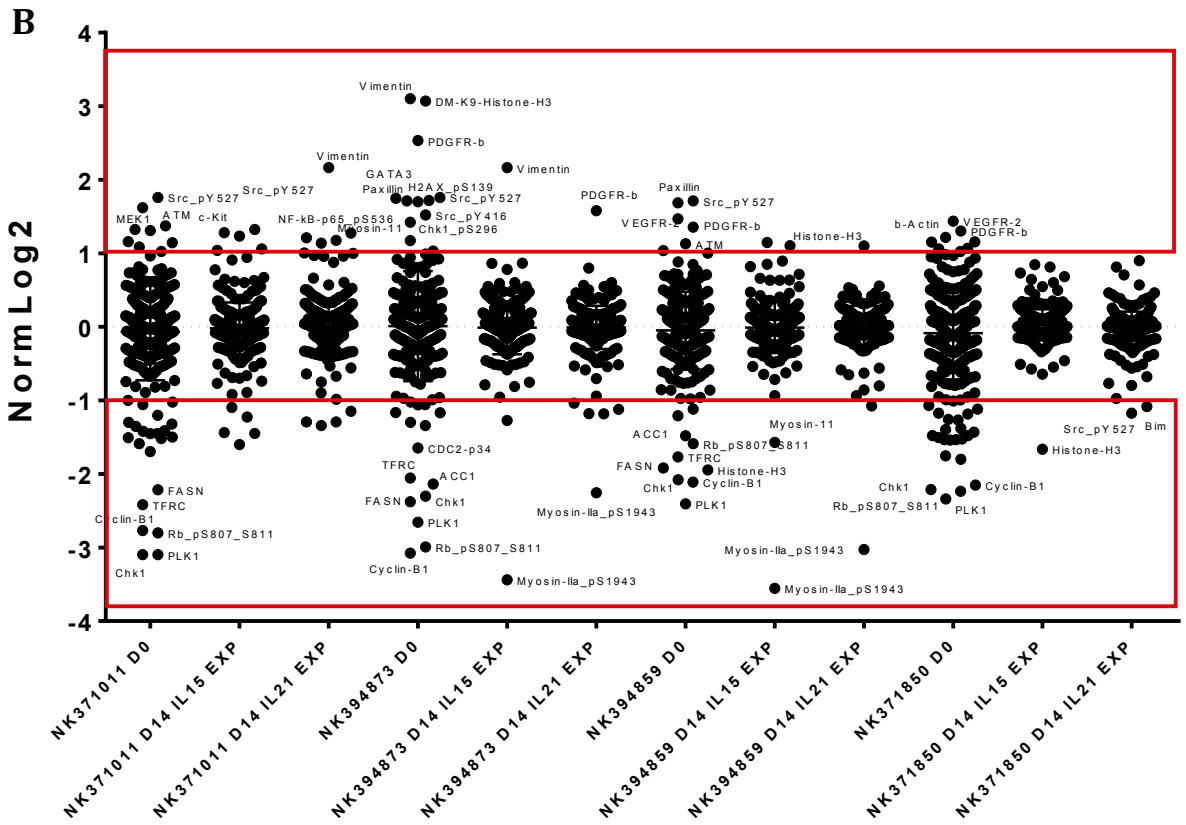


Figure 16: Protein expression overview in NK cells, before and after expansion. (A) Protein expression in NK cells was assessed by RPPA, and heat map was generated using supervised hierarchical clustering. The data shown here represents the most differentially expressed proteins between day 0 fresh and expanded NK cells in the panel. It can be observed that proteins involved in cell cycle progression, cellular proliferation and apoptosis are differentially expressed between fresh and expanded NK cells, and only a few proteins in the panel are differentially observed between mbIL15 and mbIL21 NK cells. The focus of further study and evaluation is based on this difference in protein expression and its regulation. (B) The overall protein expression was also assessed by comparison between individual donors to their expanded counterparts. Data from this assessment was pooled for statistical strength, and was used in further evaluation. Data shown here represents normalized log fold change for each of the samples tested.

We interrogated the protein expression by assessing the most differentially expressed between the 3 groups (Figure 17). Several proteins related to function in lymphocytes were differentially expressed between the groups. For instance, PDGFRβ, a receptor protein was expressed highly in fresh NK cells but was downregulated in both expanded groups. High expression of PDGFRβ in NK cells has been shown to inhibit the in vitro lytic activity of NK cells (164), suggesting that PDGFRβ expression prevents activation of NK cells under normal conditions, and hence is highly expressed in freshly isolated peripheral blood NK cells. It is also plausible that expansion causes NK cell activation, and thus PDGFRβ is downregulated in both mbIL15 and mbI21 expanded NK cells. Similar downregulation of VEGFR2 was also observed in both expansions, increased VEGFR2 is known to prevent activation of lymphocytes (165), and thus is another factor highly expressed in fresh peripheral blood NK cells, but is downregulated upon activation. Additionally, phosphorylated Src kinases were downregulated in expanded NK cells. Src kinases have redundant functions in NK cells and participate in activation of inhibitory and activating signals as well as participate in activation of MAPK and PI3K pathways (166). Other proteins included p161NK4, a protein associated with increased tumorigenesis, and c Kit involved in increased survival, proliferation and activation of mouse NK cells (167).

Additionally, although we did not find significant differences in the gene expression of Stats and TERT, we looked at the expression levels of Stats as well as phospho Stats. As expected and corresponding to mRNA data, we did not find significant differences in expression in either of the Stats or their phosphorylated proteins (Figure 17B). The RPPA panel, id not have TERT antibody, and hence expression data for TERT could not be verified.

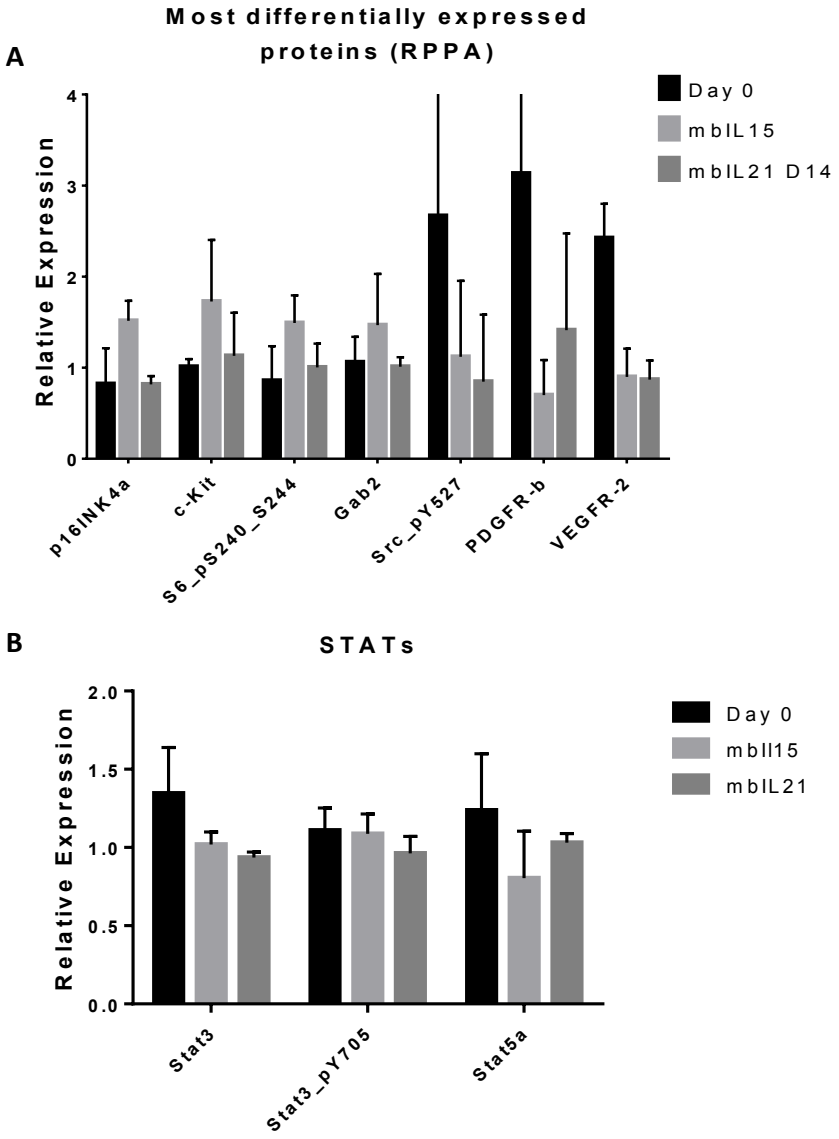


Figure 17: Comparison of most differentially expressed proteins by RPPA. (A) Proteins that were most differentially expressed ($p < 0.01$) between fresh and expanded NK cells are shown. Proteins involved in regulation of activation such as PDGFRb, VEGFR2, Src kinases and c Kit were some of the top scoring proteins. (B) Since IL-15 and IL-21 signal through Stat 5 and Stat3, respectively, we also compared the protein expression of Stat 3 and Stat5, but we did not observe significant differences in their expression ($p > 0.05$).

Next, we assessed the expression levels of proteins involved in cell cycle progression, proliferation and apoptosis between fresh and expanded NK cells (Figure 18A). We observed high expression of cell cycle proteins B1 (highest expression in mbIL21) and cyclin D1, indicating a positive correlation to increased cell cycle progression in both groups. Cyclin B1 is required for activation of mitosis and cyclin D1 plays a key role in promoting G1/S transition (168). AMP kinase A, a kinase protein involved in cellular activation from resting state, activation of glucose uptake and mitochondrial activity was upregulated in both mbIL15 and mbIL21 NK cells (higher in mbIL21 NK cells), indicating increased cellular activity and proliferation. AMP kinase A increase in NK cells has been shown to increase NK cell mediated cytotoxicity (169). Additionally, p-Akt expression was also higher in expanded NK cells, indicating an overall increase in cellular activation and metabolism.

Next, we assessed the expression levels of proteins involved in apoptosis, including both pro and anti-apoptotic proteins (Figure 18 B and 18C). We observed that most of the protein expressions were not different between fresh and expanded, or between the expanded groups, except Bim a pro-apoptotic protein encoded by BCL2L11, which was los one of the highest differentially expressed gene from mRNA analysis. The expression of Bim was high in both fresh and mbIL15 expanded NK cells, however was significantly lower in mbIL21-expanded cells, in fact Bim was the most highly differentially expressed protein between mbIL15 and mbIL21 expanded NK cells. As stated previously, high expression of Bim in peripheral blood NK cells is required for preventing NK cell proliferation under normal conditions, however, it is essential that Bim expression and corresponding induction of apoptosis is prevented in order to successfully expand NK cells.

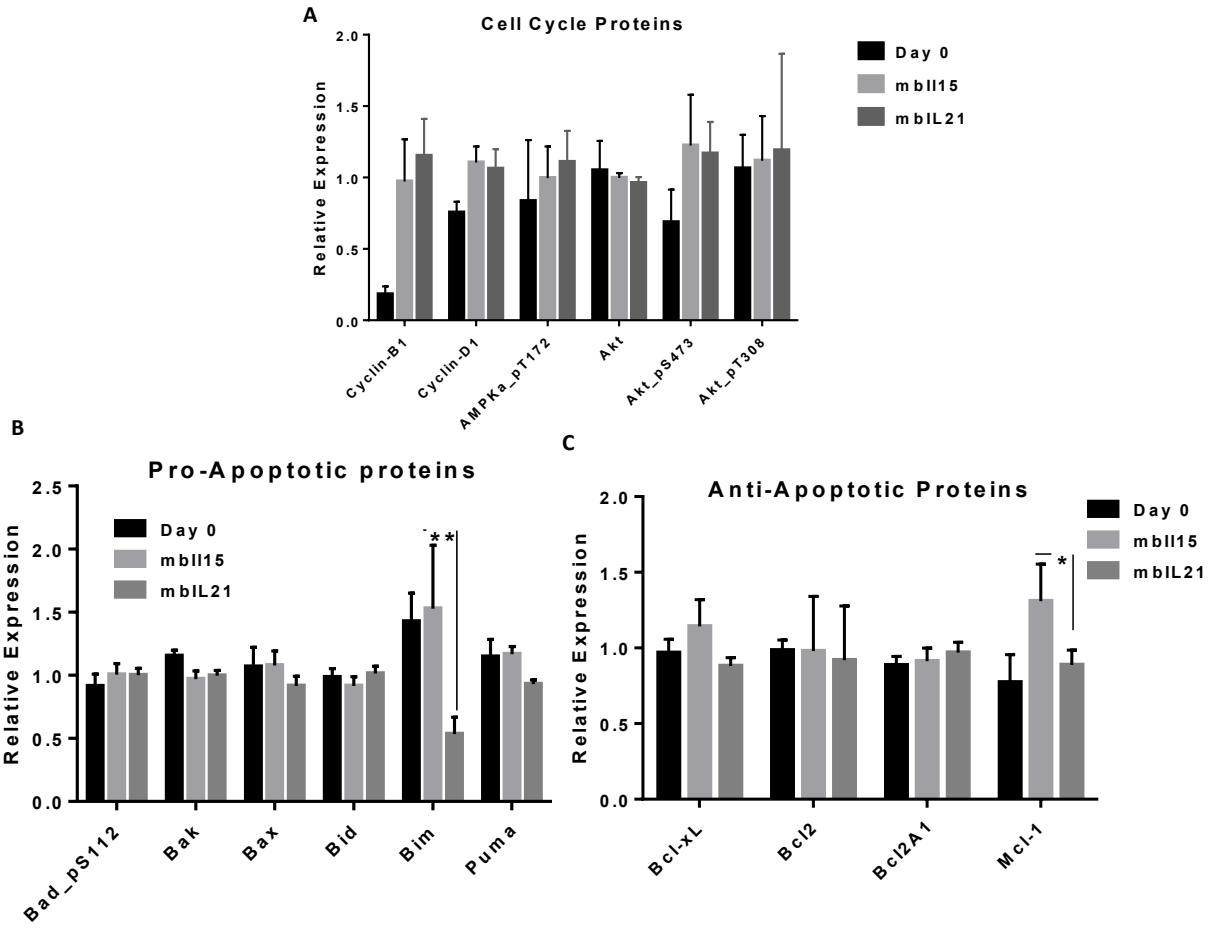


Figure 18: Expression of cell cycle and apoptotic proteins in NK cells before and after expansion. (A) Proteins involved in cell cycle progression and cellular proliferation such as cyclin D1, cyclin B1, pAKT ($p < 0.01$) and Amp kinase A ($p < 0.05$) were upregulated in both mbIL15 and mbIL21 expanded NK cells. (B) Comparison of pro-apoptotic proteins in the RPPA panel shows marginally lower expression of Bak, Bax and Puma ($p > 0.05$). As shown, the most significant and the highest differential expression between mbIL15 and mbIL21 amongst all the proteins assessed was observed with Bim ($p < 0.001$). (C) Analysis of anti-apoptotic proteins in the panel shows lower marginally higher expression of Bcl-Xl and

significantly higher Mcl-1 ($p < 0.01$) in mbIL15 expanded NK cells, however despite increased expression of these anti-apoptotic proteins NK cells expanded on mbIL15 undergo high rate of apoptosis, primarily mediated by Bim, thereby leading to an overall decreased expansion potential, when compared to mbIL21 expansion.

It is interesting to note that IL-15 signaling in vivo is associated with increased activation and proliferation of NK cells, one of the mechanisms through which IL-15 mediates this effect is by suppression of apoptosis (35). However, it is noteworthy that cytokine signaling in vivo is a tightly regulated process that allows activation or inhibition of cytolytic immune cells, only when required, and is not a continual process. This however is a remarkable contrast to activation through cytokine signaling ex vivo, where NK cells are continually stimulated by the presence of IL-15 or IL-21, in this study. It is also interesting to note that even through continual stimulation, whilst IL-21 signaling through Stat 3 caused an active downregulation of Bim, as well as maintenance of telomere length, IL-15 through Stat 5 signaling decreased telomere length, as well as increase BCL2L11 and Bim, leading to senescence and apoptotic death, respectively.

Since the differential expression of Bim due to IL-15 and IL-21 signaling is a new discovery, we also explored the mechanisms that cause the observed differences, and focused our efforts on understanding the expression profile and role of miRNA in mediating these changes.

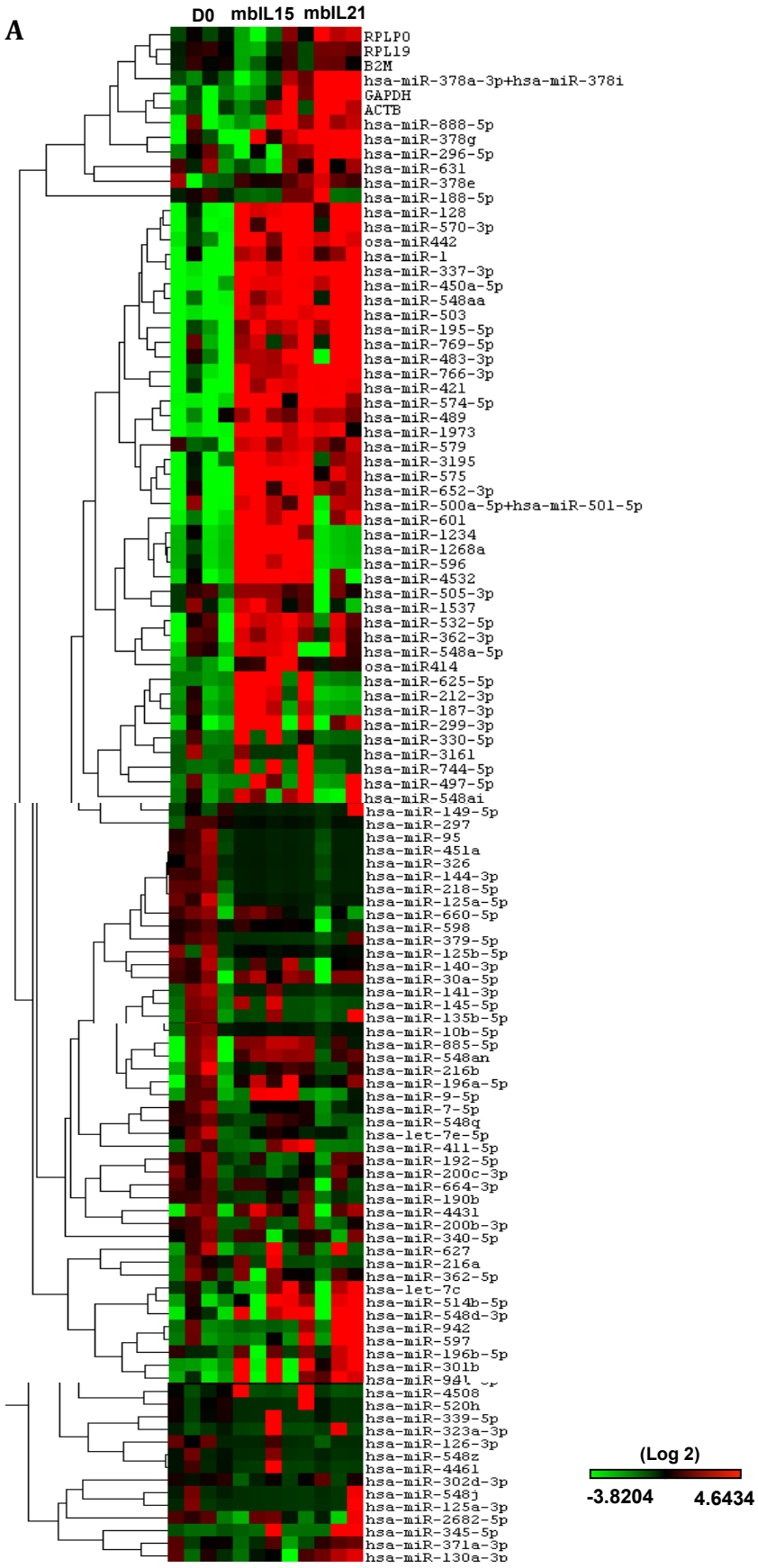
Expansion Platforms Lead To Differences In miRNA Expression Profiles

MicroRNAs are non-coding RNAs that are highly conserved and regulate numerous cellular functions including functions in immune cells. As discussed in the introduction, the role of miRNA in the development, survival, and function of T and B cells are well studied (170-173). The NK cell miRNA expression as well as the mechanisms of miRNA-regulated NK cell development, homeostasis, and functional responses is actively being investigated. The mechanisms of activation of miRNA in NK cell development, function and even diseases have been reported (131), such as the role of miR-155 in regulating interferon γ (140), miR-27a in regulation of granzyme and perforin in NK cells (174), miR-183 in regulation of TGFβ (135), to name a few. However, there are currently no studies comparing the expression of miRNA or evaluating their role in activation and expansion of NK cells or in ex vivo expansion of NK cells. Since we developed the mbIL21 platform for the expansion of NK cells, we were specifically interested in understanding the miRNA repertoire and their role in causing the prolific proliferation due to IL-21 signaling. Further, we wanted to compare the miRNA expression to the popular IL-15 cytokine based membrane bound platform, especially due to the observed differences in their expansion potential.

Based on the differential expansion capabilities of NK cells with mbIL21 and mbIL15, and the differential expression of BCL2L11, Bim, as well as our previous understanding of Stat signaling (IL-15 and IL-21 mediating Stat 5 and Stat 3, respectively), and the role of TERT in mbIL15 and mbIL21 NK cells, we hypothesized that these differences could be mediated by miRNA. We proposed that IL-15 and IL-21 signaling could potentially cause differential miRNA expression, with consequent differential function, phenotype as well as

expansion. To evaluate this hypothesis, we assessed miRNA expression in normal donor-derived peripheral blood NK cells and NK cells expanded on either mbIL15 or mbIL21 (from the same donors) by Nanostring n-Counter method. We assessed the expression of 800 miRNAs in the panel, and as expected, we observed differential expression of miRNAs between the 3 groups. Upon background correction and normalization, 117 miRNAs were found to be significantly different ($p < 0.01$) and are represented in a heat map generated by supervised hierarchical clustering (Figure 19).

We further used this data to specifically compare the miRNA expression in 2 ways. We first assessed the differential expression of miRNA in fresh NK cells compared to that of expanded (both) groups. Secondly, we assessed the most significantly differentially expressed miRNA between mbIL15 and mbIL21, and the findings from this comparison were further evaluated, as discussed below. Additionally, since the goal of this project is to specifically address the differential expansion potential between mbIL15 and mbIL21, this data is not utilized further in this specific study. However, future research based on these findings is under consideration, some of which are discussed in the future directions.


A
D0
mblL15
mblL21
RPLP0
RPL19
B2M
hsa-miR-378a-3p+hsa-miR-378i
GAPDH
ACTB
hsa-miR-888-5p
hsa-miR-378g
hsa-miR-296-5p
hsa-miR-631
hsa-miR-378e
hsa-miR-188-5p
hsa-miR-128
hsa-miR-570-3p
osa-miR442
hsa-miR-1
hsa-miR-337-3p
hsa-miR-450a-5p
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hsa-miR-574-5p
hsa-miR-489
hsa-miR-1973
hsa-miR-579
hsa-miR-3195
hsa-miR-575
hsa-miR-652-3p
hsa-miR-500a-5p+hsa-miR-501-5p
hsa-miR-601
hsa-miR-1234
hsa-miR-1268a
hsa-miR-596
hsa-miR-4532
hsa-miR-505-3p
hsa-miR-1537
hsa-miR-532-5p
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hsa-miR-299-3p
hsa-miR-330-5p
hsa-miR-3161
hsa-miR-744-5p
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hsa-miR-149-5p
hsa-miR-297
hsa-miR-95
hsa-miR-451a
hsa-miR-326
hsa-miR-144-3p
hsa-miR-218-5p
hsa-miR-125a-5p
hsa-miR-660-5p
hsa-miR-598
hsa-miR-379-5p
hsa-miR-125b-5p
hsa-miR-140-3p
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hsa-miR-145-5p
hsa-miR-135b-5p
hsa-miR-10b-5p
hsa-miR-885-5p
hsa-miR-548an
hsa-miR-216b
hsa-miR-196a-5p
hsa-miR-9-5p
hsa-miR-7-5p
hsa-miR-548q
hsa-let-7e-5p
hsa-miR-411-5p
hsa-miR-192-5p
hsa-miR-200c-3p
hsa-miR-664-3p
hsa-miR-190b
hsa-miR-4431
hsa-miR-200b-3p
hsa-miR-340-5p
hsa-miR-627
hsa-miR-216a
hsa-miR-362-5p
hsa-let-7c
hsa-miR-514b-5p
hsa-miR-548d-3p
hsa-miR-942
hsa-miR-597
hsa-miR-196b-5p
hsa-miR-301b
hsa-miR-4508
hsa-miR-520h
hsa-miR-339-5p
hsa-miR-323a-3p
hsa-miR-126-3p
hsa-miR-548z
hsa-miR-4461
hsa-miR-302d-3p
hsa-miR-548j
hsa-miR-125a-3p
hsa-miR-2682-5p
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hsa-miR-371a-3p
hsa-miR-130a-3p
(Log 2)
-3.8204
4.6434

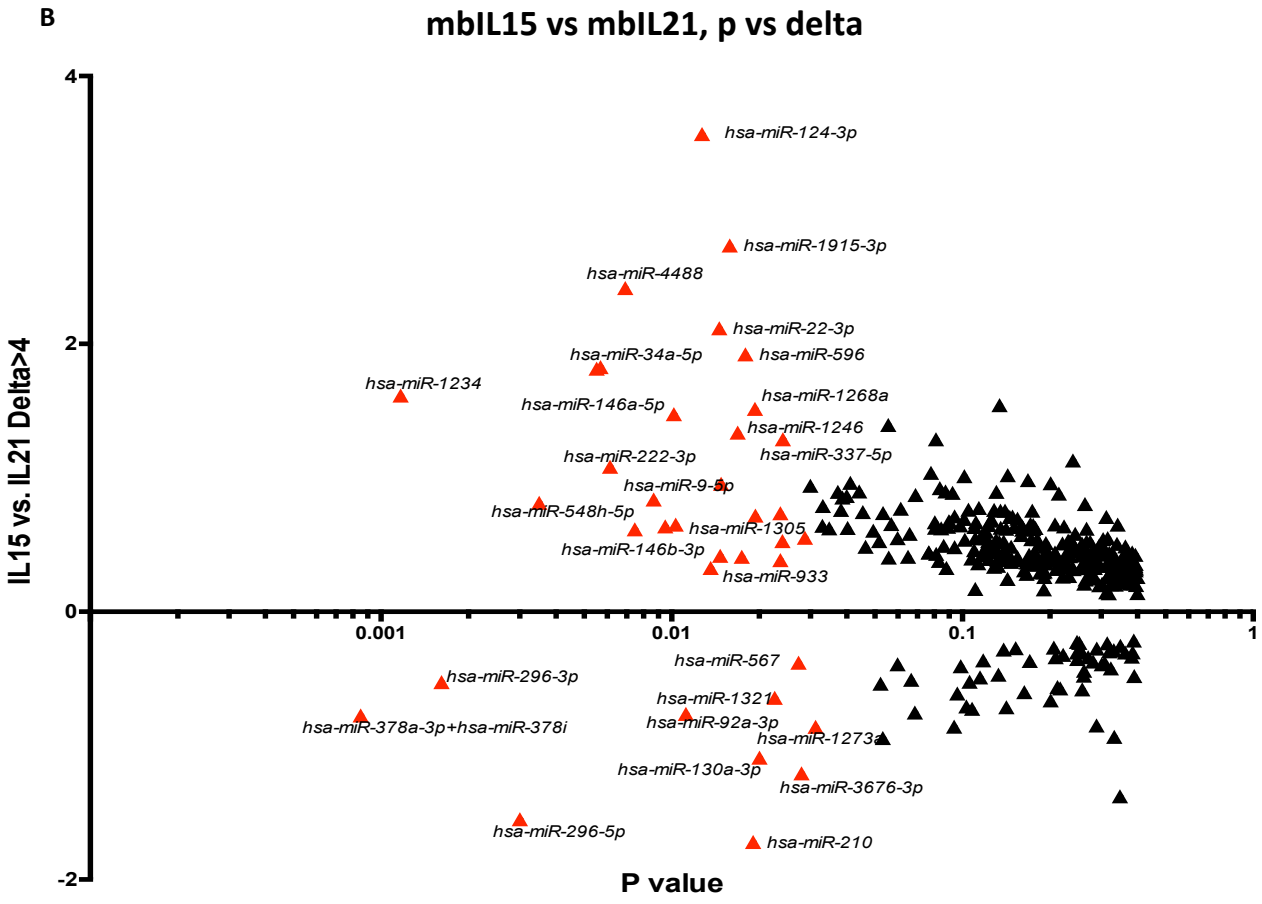


Figure 19: Differential miRNA expression between fresh, mbIL15 and mbIL21 expanded NK cells. Nanostring data for miRNA expression was assessed for 800 miRNAs, after background correction and normalization, paired student t test was performed on graphpad prism. (A) The heat map gives an overview of the number of miRNAs that are differential between fresh and expanded NK cells, as well as between mbIL15 and mbIL21. 117 of the miRNA were considered significantly differentially expressed with $p \leq 0.05$. The dataset from this heat map was further stringently assessed for difference in fold expression, and used in

subsequent experiments. (B) Volcano plot showing expression of miRNA in mbIL15 and mbIL21 expanded NK cells plotted based on significance vs. fold change with high expression in mbIL15 on top and low expression in the bottom of the panel, and vice versa for mbIL21 cells. The miRNAs indicated in red were considered significant, and similar to gene and protein expression, only a handful of the miRNAs are significantly differentially expressed based on our stringent analysis setting of $p \leq 0.01$ and fold change of delta > 4 (log2).

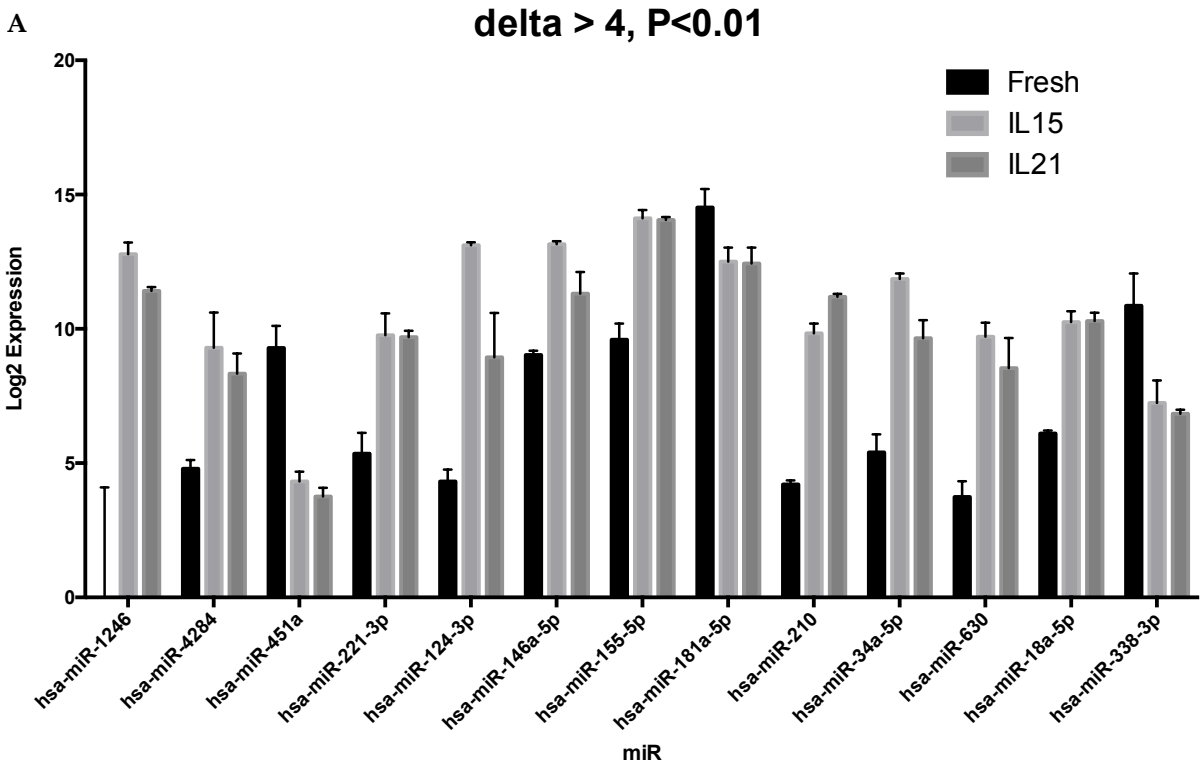
Next, we evaluated the dataset for the most stringent differential expression between fresh and expanded NK cells, by looking for miRNA that had a delta value of at least greater than 4 (8 fold difference) and our findings include13 miRNAs that fit these criteria: these miRNAs are miR 1246, miR 4284, miR 451a, miR 221-3p, miR 124-3p, miR 146a-4p, miR 1555-5p, miR 181a-5p, miR 210, miR 34a-5p, miR 630, miR 18a-5p and miR 338-5p. Interestingly, the expression pattern of these miRNA encompassed a spectrum of high and low expressing miRNA in both fresh and expanded NK cells (Figure 20A). We performed detailed correlative analyses of the most significantly differential miRNAs through search engines including miRwalk, miRbase and targetscan, to identify the significance of the miRNA to that of our protein and gene expression data. Our analyses showed that the miRNA expressed in expanded NK cells were not only regulating expansion and growth potential of NK cells, but also play significant roles in mediating critical functions in lymphocytes including NK cells. For instance, miR-1246 has been shown to be involved in B cell activation (175), miR-451a plays a role in modulating cytokine secretion in dendritic cells (176), miR 221-3p suppression increases CD4 T cell proliferation (177), miR 155-5p in IFN-γ activation (140), and miR 210 induces CD4 T cells via FOXp3 (178), to name a few validated functions. Based on the compiled information of validated and predicted targets of these miRNA as detailed on the miRwalk website (along with a comparison of 8 miRNA prediction tools), we observed that several other miRNAs in the panel have binding sites and varied functions, specifically in immune cell components (Table 5).

Further, we specifically assessed the differences in expression of miRNA between mbIL15 and mbIL21 expanded NK cells by looking for miRNAs that were expressed very highly in both mbIL15 and mbIL21 groups. Interestingly, miRNAs related to oxidative stress

response such as miR miR 34a-5p, miR 1915-3p, and miR 150-3p were comparatively downregulated in mbIL21 expanded NK cell cohort. Further, miRNAs that are involved in enhancing NK cell cytolytic functions such as let-7 family of miRs, miR 21, miR 1224 and miR 187 were equally distributed between both mbIL15 and mbIL21 expanded NK cells. However it was interesting to note that other miRNAs involved in enhancing cellular cytotoxicity related functions such as miR 27a (granzyme B and perforin), miR 155 (IFNγ), miR 146a (IRAK 1 and TRAF6: Toll like receptors; negative regulator of pro-inflammatory cytokines including TNFα), miR 125b (activation of KLF13, a transcription factor essential for T lymphocyte activation and inflammation, TNFα activation), miR 142 (IL6 activation), miR 221 (dendritic cell maturation and development), miR 378 (Granzyme B) and miR 223 (IKKα, NLRP3, Stat3 activation leading to increased immune cell differentiation, maturation, lymphocyte and granulocyte activation and IL6 production) were significantly lower in mbIL21 expanded NK cells, suggesting that the observed increased cytolytic function could be modulated by these miRNAs.

Through this study, we identified miR 124-3p to be highly expressed in mbIL15 and lower in mbIL21 by 18 fold, and to be the most differentially expressed miRNA in the group (Figure 20B). Additionally, miR 124-3p was highly expressed in fresh NK cells as well (Figure 20A).

A


delta > 4, P<0.01
Fresh
IL15
IL21
Log2 Expression
20
15
10
5
0
hsa-miR-1246
hsa-miR-4284
hsa-miR-451a
hsa-miR-221-3p
hsa-miR-124-3p
hsa-miR-146a-5p
hsa-miR-155-5p
hsa-miR-181a-5p
hsa-miR-210
hsa-miR-34a-5p
hsa-miR-630
hsa-miR-18a-5p
hsa-miR-338-3p
miR

B

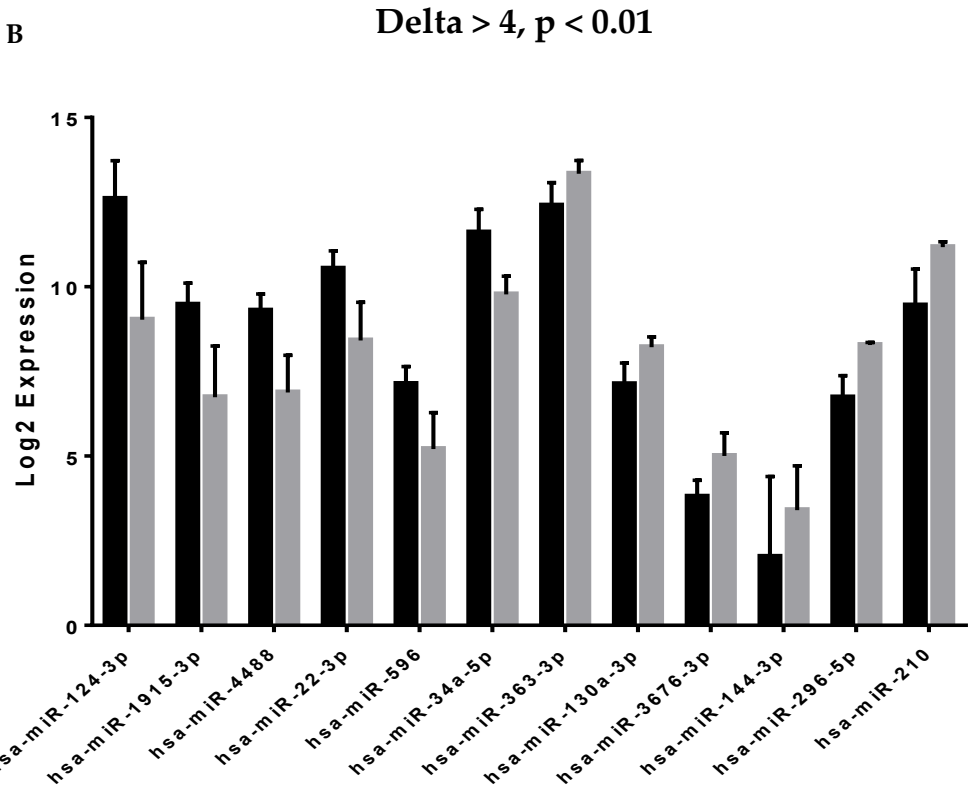

Delta > 4, p < 0.01
Log2 Expression
15
10
5
0
hsa-miR-124-3p
hsa-miR-1915-3p
hsa-miR-4488
hsa-miR-22-3p
hsa-miR-596
hsa-miR-34a-5p
hsa-miR-363-3p
hsa-miR-130a-3p
hsa-miR-3676-3p
hsa-miR-144-3p
hsa-miR-296-5p
hsa-miR-210

Figure 20: Summary of most significantly differential miRNA between fresh, mbIL15 and mbIL21 expanded NK cells. (A) The data represented here shows the most significantly differential miRNAs between fresh and expanded NK cells. As shown, miRNA that are either higher or lower in expanded cells compared to fresh NK cells are included. Data analysis was performed using paired student t test on graphpad prism, and miRNA with fold difference ≥ 8 folds were considered for further analysis. Further, we show that miR 124-3p is one of the miRNAs that are differential between fresh and expanded NK cells. (B) Differential expression between mbIL15 and mbIL21 was analyzed from the most significant dataset. We show the miRNA that are expressed highest in mbIL15 (left side of the panel) and highest in mbIL21 (right side of the panel), and corresponding expression in the other counterpart of expanded NK cells. We identified miR 124-3p to be highly expressed in mbIL1, and to be the most differentially expressed miRNA in the panel, with a 18-fold difference in expression between mbIL15 and mbIL21. Based on this finding, we further explored the role of miR 124-3p in regulation of cellular processes including proliferation and apoptosis.

	Comparitive expression levels in NK cells				
miRNA	**Fresh**	**mbIL15**	**mbIL21**	**Protein**	**Function in lymphocytes/NK cells**
miR 4284	Low	Highest	High	**CDK17**	Cell Cycle Progression
miR 451a	High	Low	Lowest	**AKT1, PIK3CA** **GATA3** **JAK2**	Signaling activation of NKG2D Maturation of NK cells, IFN-γ production IFN-γ signaling and activation
miR 221-3p	Low	High	High	**CDK1B, CDK1A, CDK6** **CCND1**	Cell Cycle Progression Cellular proliferation
miR 124-3p	High	Highest	Low	**STAT3** **CCND1** **CDK6, CDKN2D, CDK4, CDKN1B** **CD28**	IL-21 signaling, diffreentiation, proliferation, telomere length Cellular proliferation Cell Cycle Progression Co-activating receptors for NK cells
miR 146a-5p	High	Highest	Low	**IL-1** **IL-6** **IL-7** **IL-8** **STAT1** **CDKN2A** **IFN-γ, TNF**	Costimulates IFN-γ production NK cell production NK cell survivsl T cell recruitment IL-15 signaling Cell Cycle Progression Cytolytic function
miR 155-5p	Low	High	High	**IL-10**	Cytotoxicity
miR 181-5p	High	Low	Low	**ZAP70** **DAP12**	Cellular activation of ADCC NKp44 activation, co-receptor for activation
miR 1915-3p	Low	High	Low	**Dicer** **Notch1** **BCL2**	Increases survival and activation Increases generation of functional NK cells Anti-apopttic protein
miR 4488	Low	High	Low	**TNF-α** **IL-6**	Inflammation and immune system development NK cell production
miR 22-3p	High	High	Low	**PTEN** **IL-1** **AKT** **Dicer**	Regulation of imune response, autoimmunity Costimulates IFN-γ production Increases cellular activity ad metabolism Increases survival and activation
miR 34a-5p	Low	Highest	High	**Notch1** **CDKN1A, CDK6, CDKN2A** **CCND1**	Increases generation of functional NK cells Cell Cycle Progression Cellular proliferation
miR 210	Low	High	Highest	**Notch1** **DICER** **STAT5A** **CDKN1A, CDKN2A,** **CCND1**	Increases generation of functional NK cells Increases survival and activation IL-15 signaling Cell Cycle Progression Cellular proliferation
miR 144-3p	Highest	Low	Low	**MTOR**	Induces proliferation and apoptosis

Table 5: Correlation of differentially expressed miRNA to their known regulatory functions. The miRNA that were differentially expressed were correlated for their known function based on literary evidence for validated targets in various cell types including NK cells and other lymphocytes. We identified several miRNA functions related to cell cycle progression, cellular proliferation, apoptosis and cytolytic function. The direction of correlation in the expression of miRNA to the activation or suppression status of specific proteins, in NK cells is not compared in this table. Plans for future studies based on miRNA-mediated regulation of NK cell activation, proliferation and function is possible from this dataset.

MiR 124-3p

MiR 124-3p belongs to the 124 family of miRNAs, and originally characterized in neuronal cells where it is expressed abundantly (179). Not much is known about the role of miR 124-3p in immune cells, except recent reports in dendritic cell development, regulation of M2 macrophage polarization and T(H)1 and T(H)2 helper T cell development (180). However, miR 124-3p is extensively studied in tumor cells and has been shown to impact proliferation, apoptosis and invasive potential in these cells (181). Under normal conditions, miR 124-3p is highly expressed acts as a tumor suppressor, but is deregulated in several tumor types including breast cancer, colorectal cancer, nasopharyngeal carcinoma, glioblastoma, MDS and prostate cancer and hepatocellular carcinoma (182-186). Downregulation of miR 124-3p in these tumors has been shown to increase proliferation and decrease apoptosis. Interestingly, miR 124-3p has a 3'UTR binding site for STAT 3, and has been shown to downregulate STAT-3 as well as affect phosphorylation of STAT 3, thereby its function in inducing proliferation and prevention of apoptosis (187). Additionally, miR 124-3p has a binding site for BCL2L11, however, activation of miR 124-3p has also been shown to induce apoptosis in cells through several mechanism including suppression of STAT 3, suppression of CCND2, MMP2 and inhibition of anti-apoptotic proteins (188). Additionally recent evidences show that miRNAs could potentially switch their roles from repression to activation depending on the cellular stimuli (112). Furthermore, it is interesting to note that miR 124-3p has a potential binding site for TERT, and could also regulate TERT (189), in addition to STAT 3 and inducing apoptosis.

Correlation Of Key Findings

In our studies, we observed similar findings with mbIL15 and mbIL21 expansions of NK cells. W observed that mbIL15 expanded NK cells showed higher miR 124-3p expression, and lower expansion, as well as increased apoptosis, as explained previously. On the other hand, we also observed that miR 124-3p levels were comparatively lower in mbIL21 expanded NK cells, and expansion potential was higher and apoptosis was lower in these cells. Additionally, we reported differential expression of BCL2L11 and Bim through this study, as well as the role of STAT 3 and TERT in causing the differential expansion between mbIL15 and mbIL2 1through our previous findings.

This kindled our curiosity to study miR 124-3p, Bim, Stat 3 and TERT in the context of NK cell expansion, and evaluate the role of miR 124-3p in modulating IL-15 and IL-21mediated expansion of NK cells. We hypothesized that *upregulation of miR 124-3p is responsible for diminished expansion of NK cells on membrane bound IL15 compared to membrane bound IL21 due to decrease in Stat 3 and increase in Bim (BCL2L11) leading to reduced proliferation and increased apoptosis.* In order to address this, we proposed that knockdown of miR 124-3p, followed by expansion with mbIL15 would increase Stat 3, decrease Bim and restore the proliferation potential and reduce apoptosis, thereby increasing the overall expansion in these cells, compared to mbIL21 expanded NK cells (Figure 21).

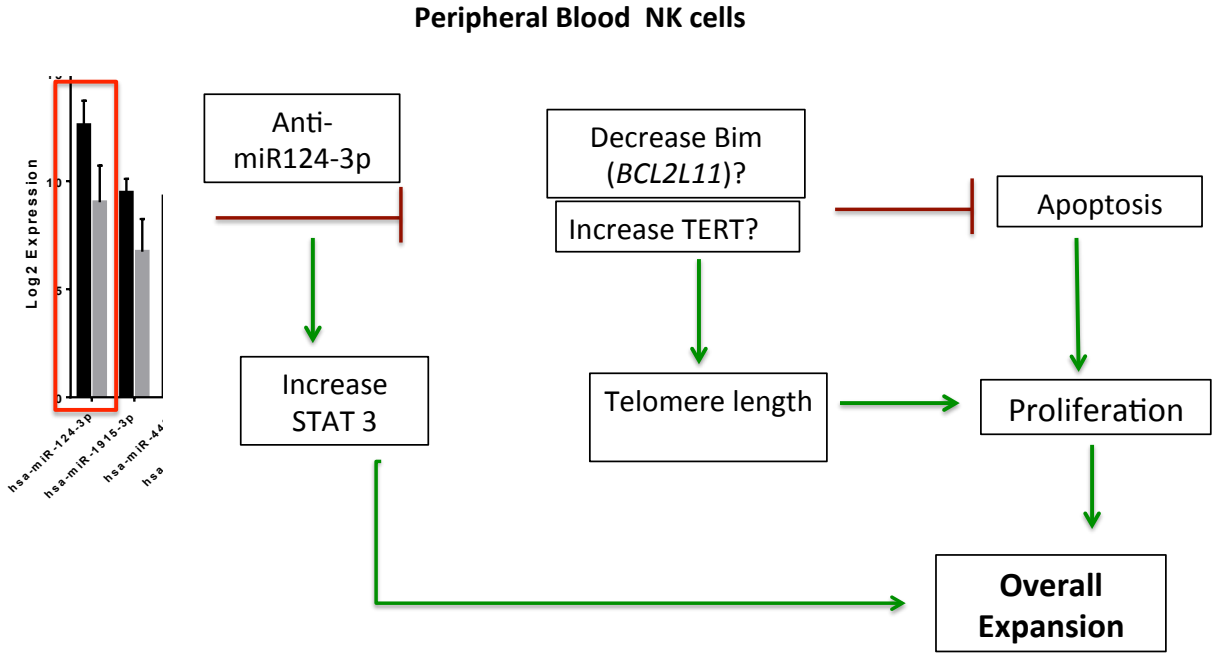


Figure 21: Schematic of correlation of key findings miR 124-3p, BCL2L11 and Bim.

Through the global expression analysis, we identified that miR 124-3p as the most differentially expressed miRNA between mbIL15 and mbIL21. Additionally, we identified differential expression of BCL2L11 and Bim. Combining this with our previous findings on STAT3 and TERT, we proposed to knockdown the expression of miR 124-3p in fresh NK cells, prior to expansion, and evaluate its effect on apoptosis, proliferation, TERT expression as well as overall expansion with mbIL15. We propose that knockdown of miR 124-3p would have beneficial effect of increasing expansion of NK cell by decrease in apoptosis and increase in proliferation. Additionally, this validation would render mechanistic insight into one of the methods by which IL-15 and IL-21cytokine signaling in NK cells is being modulate by miRNA, specifically miR 124-3p, in causing a prolific expansion differential.

Mir 124-3p Regulates Proliferation, Apoptosis And Senescence In NK Cells

To assess the potential of miR 124-3p in regulating proliferation, apoptosis and senescence in NK cells and its role in regulating the overall differential expansion of NK cells, we performed transient knockdown of miR 124-3p in NK cells. We preferred the transient knockdown method for the following reasons: Firstly, the entirety of this work is based on primary NK cells, and these cells are not easily amenable to genetic modifications, and hence technically challenging to achieve a permanent knockout model. Secondly, we have established techniques in our lab to successfully transiently knock down proteins with siRNA previously, and have reported that primary NK cells tolerate transient knockdown reasonably (190). Based on this, we knocked down miR 124-3p in normal donor-derived and Rosettesep-purified fresh peripheral blood NK cells. The effect of knock down was assessed for all subsequent experiments by measurement of miR 124-3p by qRT-PCR.

As detailed in the methods section, fresh NK cells were electroporated with anti-miR 124-3p along with negative controls. Unelectroporated cells were used as baseline. Since we used primary samples for all experiments, and electroporated the cells, we worked with smaller sample size, and hence we followed the expression of miR 124-3p for 4 days. We observed that the peak knockdown effect of miRNA was between 24 and 48 hours, following which miR 124-3p increased in NK cells, and was completely normalized by day 4. We also observed a 2.4 fold decrease in the expression of miR 124-3p (Figure 22A and 22B).

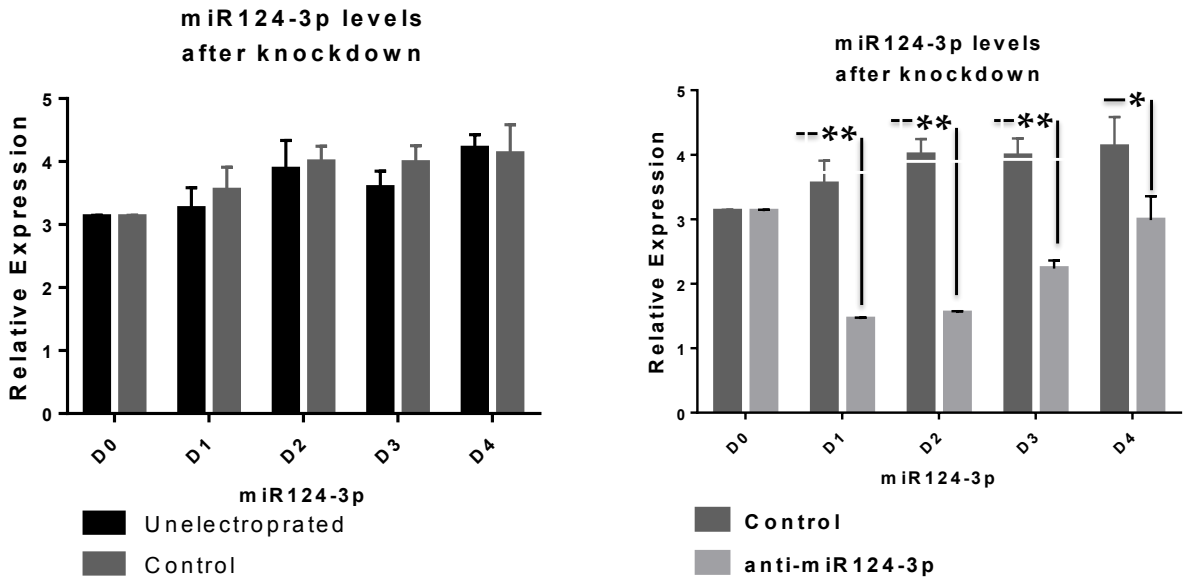


Figure 22: miR 124-3p expression in NK cells following knockdown by electroporation. Fresh NK cells were electroporated with 200 nM of anti-miR 124-3p or negative control using Amaxa nucleofector (data shown for n=4 electroporation). Electroporated cells were rested overnight in complete RPMI media freshly supplemented with IL-2, and miR 124-3p was measured by Taqman miRNA q RT-PCR. Statistical analysis was performed on Graphpad prism by student t test, * indicates $p < 0.05$, ** indicates $p < 0.01$. (A) Comparison of miR 124-3p between unelectroporated and negative control electroporated NK cells. As shown, electroporation with negative control did not alter miR 124-3p expression in NK cells. (B) Comparison of anti-miR 124-3p and negative control electroporated NK cells indicate that miR 124-3p is significantly reduced after knock down (2.4 folds after 24 hrs). The effect of knockdown was normalized by day 4 in culture, and hence subsequent correlative gene and protein expressions were measured within this time frame.

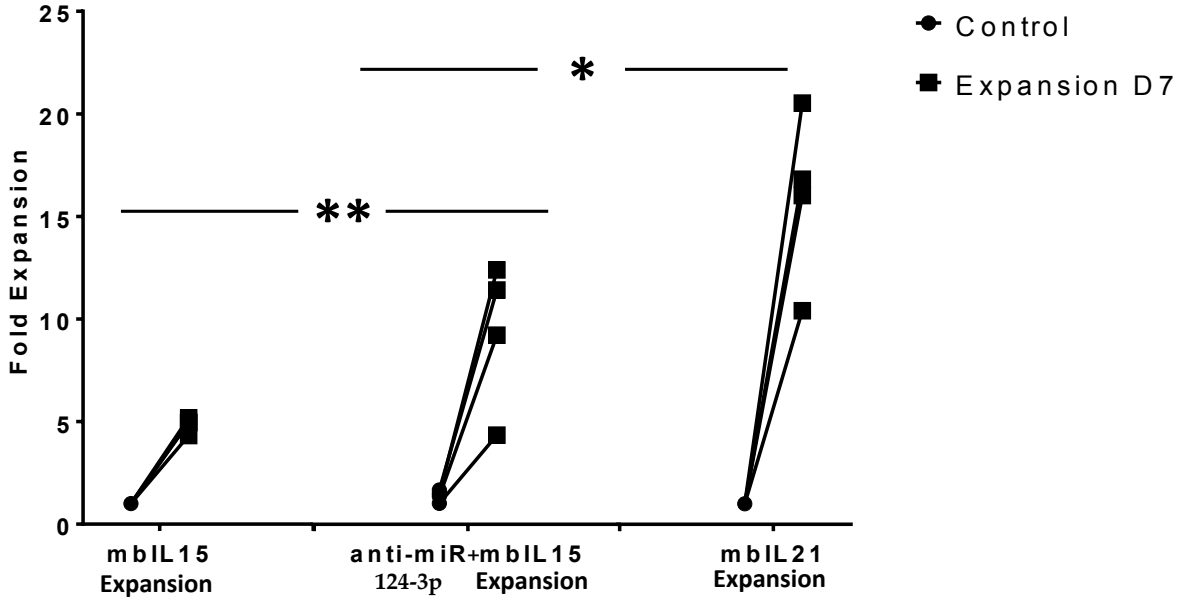
Mir 124-3p Knockdown Increases Overall Expansion

Upon confirmation of successful transient knockdown of miR 124-3p in NK cells, we assessed the impact of miR 124-3p knockdown on the overall expansion of NK cells. Normal donor-derived and Rosettesep purified NK cells were electroporated with anti-miR 124-3p or negative control as detailed above. Unelectroporated NK cells served as baseline comparative control. All 3 fractions of NK cells were divided into 2 parts, and expansion was set up with either K562.mbIL15 or K562.mbIL21, as described previously. After 7 days, NK cells were counted by trypan blue exclusion method, as well as assessed by CD56, CD16, NKp46 and lack of CD3 expression by flow cytometry. The fold expansion of these NK cells was calculated as described in detail in the methods section. The fold expansion comparison between the 3 groups (unelectroporated, negative control and anti-miR 124-3p electroporated cells) is represented in Figure 23.

As shown in Figure 23, the overall expansion of NK cells with mbIL15 was improved significantly, and was comparable to mbIL21 expanded NK cells. Additionally, the effect of knockdown of miR 124-3p was more prominent in mbIL15 than in mbIL21 expansion, possibly due to the robustness of mbIL21 expansion due to an existing lower expression of miR 124-3p in these cells (data not shown).

By knocking down miR 124-3p, we were able to demonstrate improved expansion of NK cells with mbIL15, although not to the extent of IL-21 mediated expansion, primarily due to 2 reasons: firstly, we compared electroporated and mbIL15 expanded NK cells to that of unelectroporated and mbIL21 expanded NK cells in order to show the difference in improved

expansion. It is possible that anti-miR 124-3p and mbIL15 expanded cells had to overcome electroporation-induced stress before expansion potential could be wholly realized. Additionally, knockdown of miR 124-3p in this situation is transient, and thus it could be argued that a potential knockout (such as CRISPR or lentiviral transduction) would be more beneficial, since miR 124-3p expression will be completely eliminated, and thus expansion could be unimpeded even with mbIL15. Further this effect on the overall improvement in efficacy of mbIL15 mediated expansion lays credence to our hypothesis, and to the potential role of miR 124-3p in regulating and driving the overall expansion potential of NK cells.



* indicates p<0.05

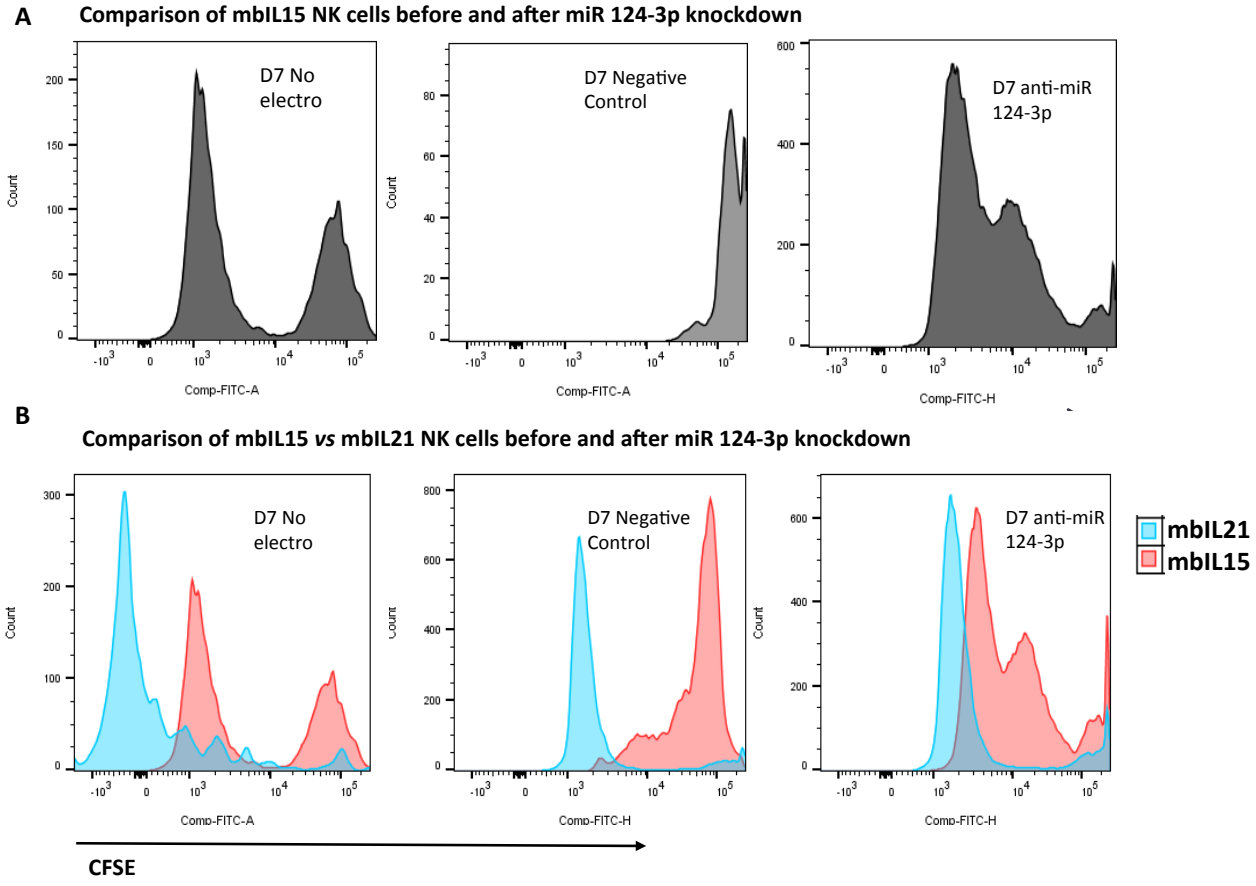
** indicates p<0.01

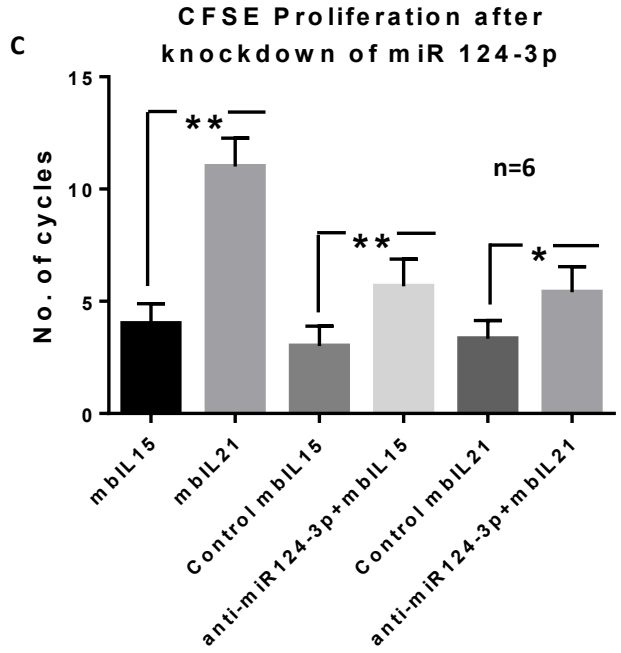
Figure 23: Knockdown of miR 124-3p enhances expansion of mbIL15 NK cells. NK cells from 4 normal donors were expanded with mbIL15 (left panel), as shown, cells expanded at an average 5-folds in 7 days. NK cells form the same donors were expanded on mbIL21 as well (right panel), with an average 17-fold expansion (difference in fold expansion $p < 0.01$). NK cells were also subject to miR 124-3p knockdown by electroporation and expanded on mbIL15 as shown (middle panel). After 7 days, fold expansion in mbIL15 increased to an average of 9.75 folds ($p < 0.05$, compared to mbIL21 expansion). This data substantiates the role of miR 124-3p in mediating the increase in overall expansion potential of NK cells.

Mir 124-3p Knockdown Increases Proliferation

To further understand the process by which miR 124-3p knockdown causes this increase in expansion, we evaluated if proliferation increased in NK cells upon miR 124-3p knockdown. Under tumor conditions, decrease in miR 124-3p has been previously shown to increase proliferation of tumor cells by activation of STAT 3, cyclin dependent kinase 6, Rb gene as well as activation of anti-apoptotic proteins (BCL2, MCL1) (181, 183-185, 187, 191). Normal donor-derived and Rosettesep purified fresh NK cells were electroporated with anti-miR 124-3p and negative control as before. Corresponding unelectroporated NK cells were used as baseline comparative control. All 3 fractions of NK cells were pre-loaded with CFSE dye, followed by expansion with either mbIL15 or mbIL21. The rate of proliferation was measured by flow cytometry on day 1, 4 and 7, by assessing the number of proliferative cycles on $CD56^{+}NKp46^{+}$ NK cells (Figure 24).

We compared the miR 124-3p knockdown NK cells' proliferation with that of negative control, as well as mbIL21 expanded NK cells, and observed an increase in the number of NK cell proliferative cycles as measured by flow cytometry. Further, we observed that the effect of knockdown of miR 124-3p was more significant in mbIL15 expansion, compared to that of mbIL21 expansion. Additionally, similar to the overall expansion pattern, despite NK cells having to overcome the electroporation-induced stress, they nevertheless were able to proliferate better when compared to unelectroporated mbIL15 NK cells. This data shows that miR 124-3p is involved in mediating the proliferative potential in NK cells and is differential between mbIL15 and mbIL21, due their differential expression.





* indicates p<0.05

** indicates p<0.01

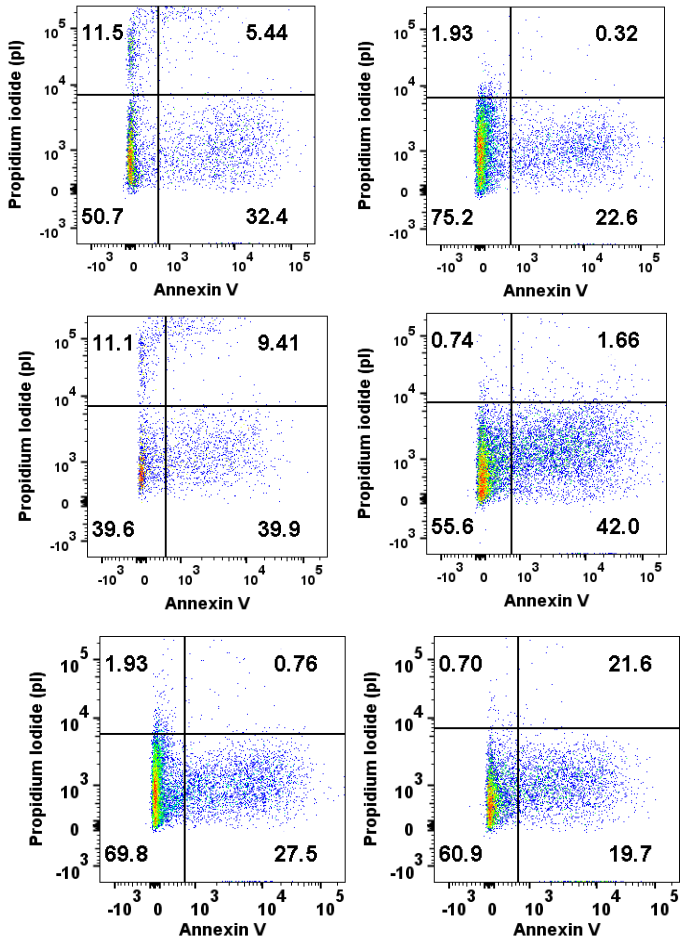
Figure 24: Knockdown of miR124-3p enhances proliferation in mbIL15 NK cells. NK cells from 6 donors were electroporated with anti-miR 124-3p and pre-loaded with CFSE dye to evaluate proliferation. NK cells were then expanded on either mbIL15 or mbIL21 and assessed for proliferation by flow cytometry on day 1, day 4 (data not shown) and day 7. Although NK cells were pre-loaded with CFSE, $CD56^{+}NKp46^{+}$ cells were gated to ensure accuracy. Data shown here is representative of 6, bar graph shows average and median centric analysis of the number of proliferative peaks between the different groups. (A) Comparison of CFSE proliferation between unelectroporated, negative control and miR 124-3p knockdown NK cells that were expanded on mbIL15. As can be seen, mbIL15 expansion did not promote proliferation of NK cells (left panel), however, miR 124-3p knockdown, supports better proliferation with mbIL15 ($p < 0.05$). (B) Comparison of proliferation between unelectroporated, negative control and miR 124-3p knockdown cells that were expanded with mbIL15 and mbIL21. Data is representative from the same donor. As shown, miR 124-3p knockdown enhanced proliferation in mbIL15 NK cells, indicating the role of miR 124-3p in promoting proliferation in NK cells ($p < 0.01$). (C) The number of proliferative peaks for each sample was counted and the data shows the average number of proliferative cycles in each group. As expected, mbIL21 is more robust in supporting proliferation (left panel), however, miR 124-3p knockdown removed the proliferative check due to continual IL-15 signaling, and promotes proliferation even in mbIL15 NK cells. P values were calculated by paired student t test on graphpad prism, and significance is indicated in the bar graph.

Mir 124-3p Knockdown Decreases Apoptosis

Mir 124-3p has been shown to be high under normal conditions, and acts as a tumor suppressor, but has been shown to be downregulated in several cancers, and one of the primary ways by which miR 124-3p exerts its action is by the inhibition of STAT 3, increased proliferation and decreased apoptosis in tumor cells (181, 184, 192). Based on this, we showed that knockdown of miR 124-3pand stimulation with mbIL15 increased overall expansion in NK cells due to increased proliferation. In addition, we also evaluated the effect on miR 124-3p knockdown on the overall apoptosis by flow cytometry. Similar to the expansion and proliferation analyses, we knocked down miR 124-3p in 3 normal donor-derived and Rosettesep purified NK cells, and expanded the cells with mbIL15 or mbIL21. We assessed the effect of knockdown by flow cytometry on day 4 and day 7 (day 4 data not shown), by staining for Annexin V and PI and gated for NK cells on $CD56^{+}NKp46^{+}$ population (Figure 25). Unelectroporated NK cells were also expanded with mbIL15 and mbIL21, and as expected mbIL15 NK cells had increased apoptotic and lower live cell populations (Figure 15).

Based on the flow cytometry data, we observed that knockdown of miR 124-3p had a significant effect on lowering the apoptotic rate in mbIL15 expanded NK cells. As before, we also observed that the effect was more pronounced in mbIL15 expansion compared to mbIL21. This could be because of the fact that the apoptosis was already very low in mbIL21 expansion. Furthermore, this data shows that miR 124-3p is involved in mediating the apoptosis in NK cells and, lowering miR 124-3p has beneficial effects to the overall expansion potential of NK cells.

A

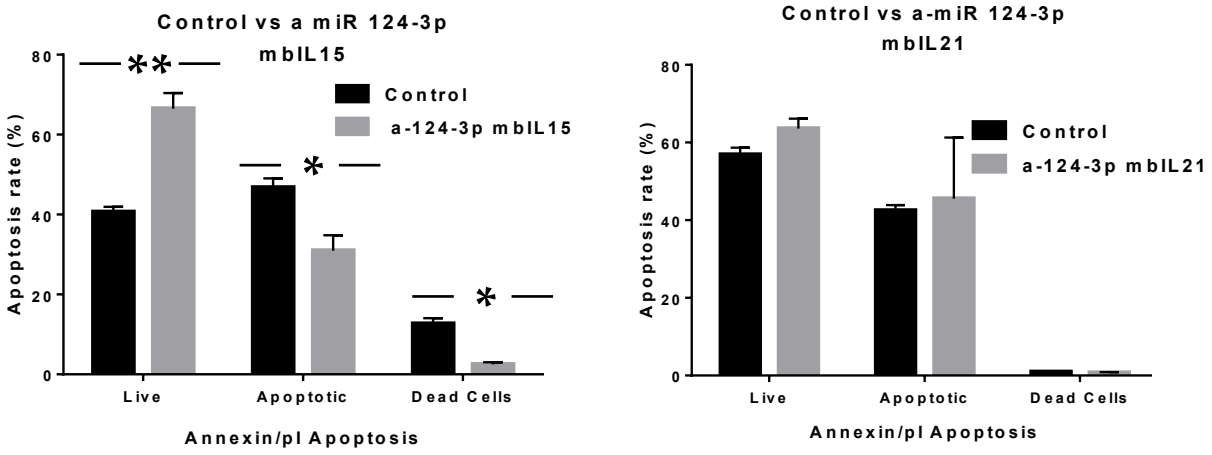


Unelectroporated NK cells mbIL15 (left) vs mbIL21 (right)

Negative Control electroporated NK cells mbIL15 (left) vs mbIL21 (right)

miR124-3p knockdown NK cells mbIL15 (left) vs mbIL21 (right)

B



* indicates p<0.05

** indicates p<0.01

Figure 25: Knockdown of miR124-3p reduces apoptosis in mbIL15 NK cells. NK cells were tested for the rate of apoptosis before and after knockdown of miR 124-3p. Fresh peripheral blood NK cells from 3 donors were electroporated with negative control or anti-miR 124-3p, followed by expansion with either mbIL15 or mbIL21. Unelectroporated NK cells were use as baseline control and the combined data on apoptosis of unelectroporated NK cells is shown in Figure 15. Percentage apoptotic, live and dead cells were calculated based on the flow cytometry data, and analyzed on graphpad prism by paired student t test, and significance are indicated in the bar graph. (A) Representative flow plots are shown. NK cells were expanded for 7 days following knockdown of miR 124-3p and apoptosis was assessed by Annexin V and PI positive cells gated on CD56 and NKp46. The top panel represents comparison between unelectropoareted mbIL15 and mbIL21 NK cells, as shown mbIL15 shows higher rate of apoptosis. A similar trend is observed in negative control NK cells as well. However, expansion following knockdown of miR 124-3p (bottom flow panel) indicates that NK cells are able to overcome the continual IL-15 signaling mediated apoptosis. (B) Comparison of negative control and miR 124-3p knockdown cells indicates that mbIL15 expansion of NK cells after miR 124-3p knockdown are able to prevent apoptosis, and increase the percent live cells. This effect was not as pronounced in mbIL21 NK cells where the rate of apoptosis was lower in both negative control as well as miR 124-3p knockdown cells (right panel).

Mir 124-3p Knockdown Regulates Expression of STAT 3, TERT And BCL2L11 (Bim)

Through our experiments, we had validated role of knock down of miR 124-3p in increasing proliferation, decreasing apoptosis, and as a result increasing the overall expansion potential of NK cells. As stated previously, miR 124-3p has been shown to promote tumorigenesis through downregulation of STAT3. We have also shown through our expression profile studies that BCL2L11 and Bim were the most significantly differentially regulated gene and protein, respectively, when compared between mbIL15 and mbIL21 NK cells, and that miR 124-3p is the most significantly differential miRNA between the 2 groups. Additionally, we have shown through previous studies that STAT3 (IL-21) signaling in NK cells promotes telomere maintenance, whereas STAT 5 (IL-15) signaling prevents TERT expression, and by extension, telomere length.

In order to corroborate our findings in this study, as well as to confirm the role of STAT3, TERT and BCL2L11 in NK cells, we proposed to knockdown miR 124-3p in fresh NK cells and follow the expression changes in these genes. Additionally, although STAT3, STAT5 and TERT were shown to be critical to the expansion with IL-15 and IL-21 in previous studies, we did not find the expression levels of these genes to be different through gene expression studies (Figure 14). Hence, we wanted to capitulate the effect of knockdown of miR 124-3p at the earliest possible timeline, and hence chose to perform the experiments on fresh peripheral blood NK cells. We electroporated the NK cells with either negative control or anti-miR 124-3p, and followed the gene expression of STAT3, TERT and BCL2L11 on day 1, 2 and 3 (as much as the sample size permitted) by qPCR.

We observed that the expression levels of STAT3 and TERT increased significantly. More interestingly, the expression of BCL2L11 significantly reduced, indicating that although miR 124-3p has a 3' UTR binding site for BCL2L11, suggesting that the activation or repression of expression is context dependent based on cellular stimuli (Figure 26). Additionally, two co-expressed mRNA transcripts that are targeted by the same miRNAs are functionally coupled to one another as a result of the finite amount of available miRNA. Thus, transient changes in the expression levels of one of the genes will have a direct or indirect impact on the expression and abundance of the other transcript driven by the amount of miRNA availability (112, 120).

Further, we assessed the effect of IL-15 and IL-21 signaling in modulating the expression levels of STAT3, TERT and BCL2L11 after miR 124-3p knock down. We electroporated fresh NK cells with negative control or anti-miR 124-3p, and expanded the cells for7 days with mbIL15 and mbIL21. The NK cells were FACS sorted on day 7 based on CD56 and NKp46 expression, and mRNA expression was evaluated by qPCR. We observe that despite mbIL15 expansion and IL-15 mediated signaling in NK cells, knockdown of miR 124-3p increased STAT3 and TERT significantly, and reduced BCL2L11 significantly (Figure 27). This data suggests that miR 124-3p mediates its effect on expansion by increasing proliferation, decreasing senescence and decreasing apoptosis, specifically by modulating the expression of STAT3, TERT and BCL2L11.

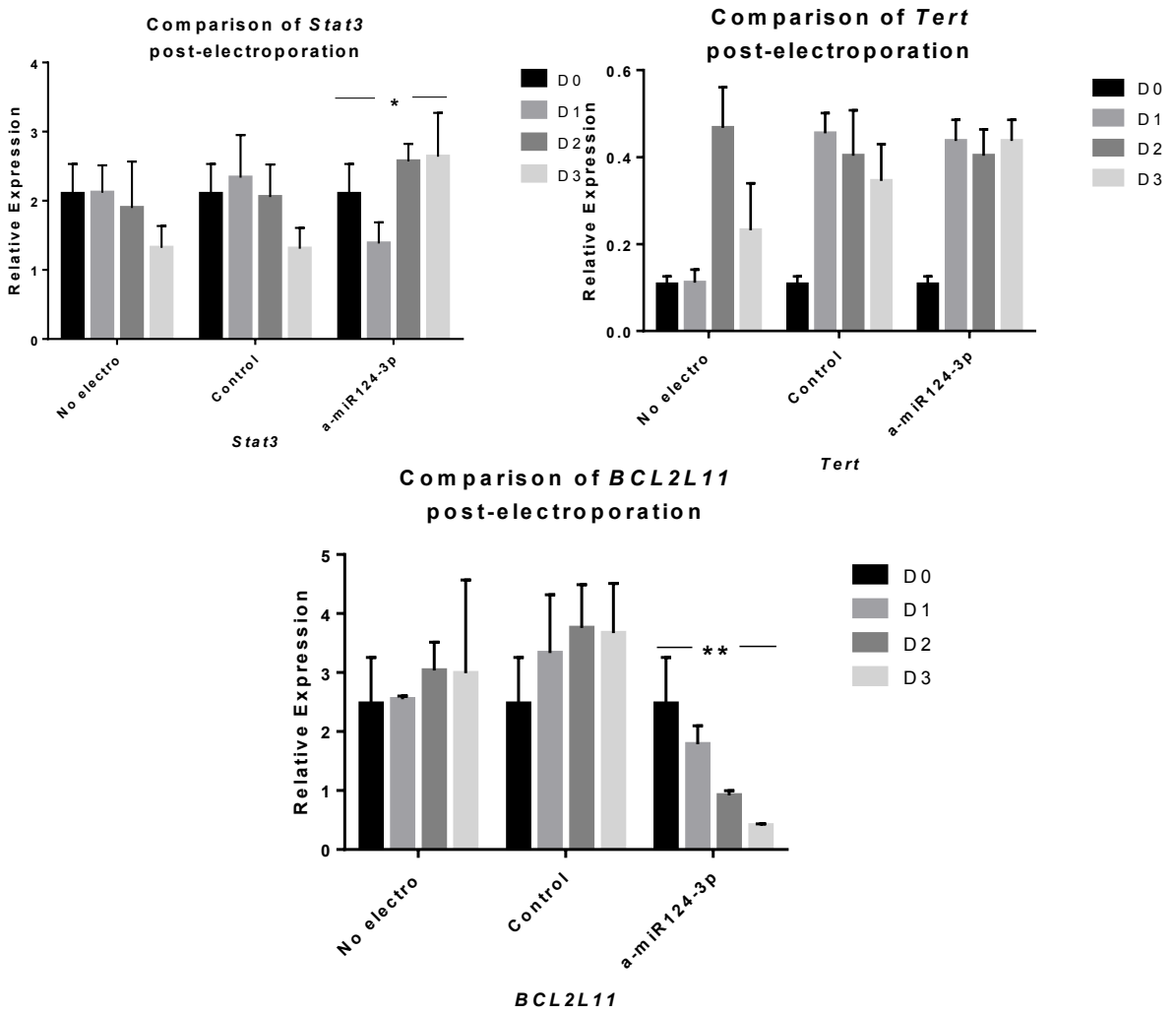


Figure 26: Knockdown of miR124-3p increases STAT3 and TERT, and decreases BCL2L11 in NK cells. Fresh peripheral blood NK cells were electroporated with negative control or anti-miR 124-3p, and unelectroporated NK cells were used as baseline comparative controls. mRNA was collected from collected from the cells 1 2 and 3 days after electroporation and gene expression was evaluated by qRT-PCR for the expression of STAT3, TERT and Bim. As shown, $\Delta\Delta C_t$ values for the expression levels of STAT3 and TERT increased significantly over 3 days, whereas BCL2L11 decreased following knockdown (n=3).

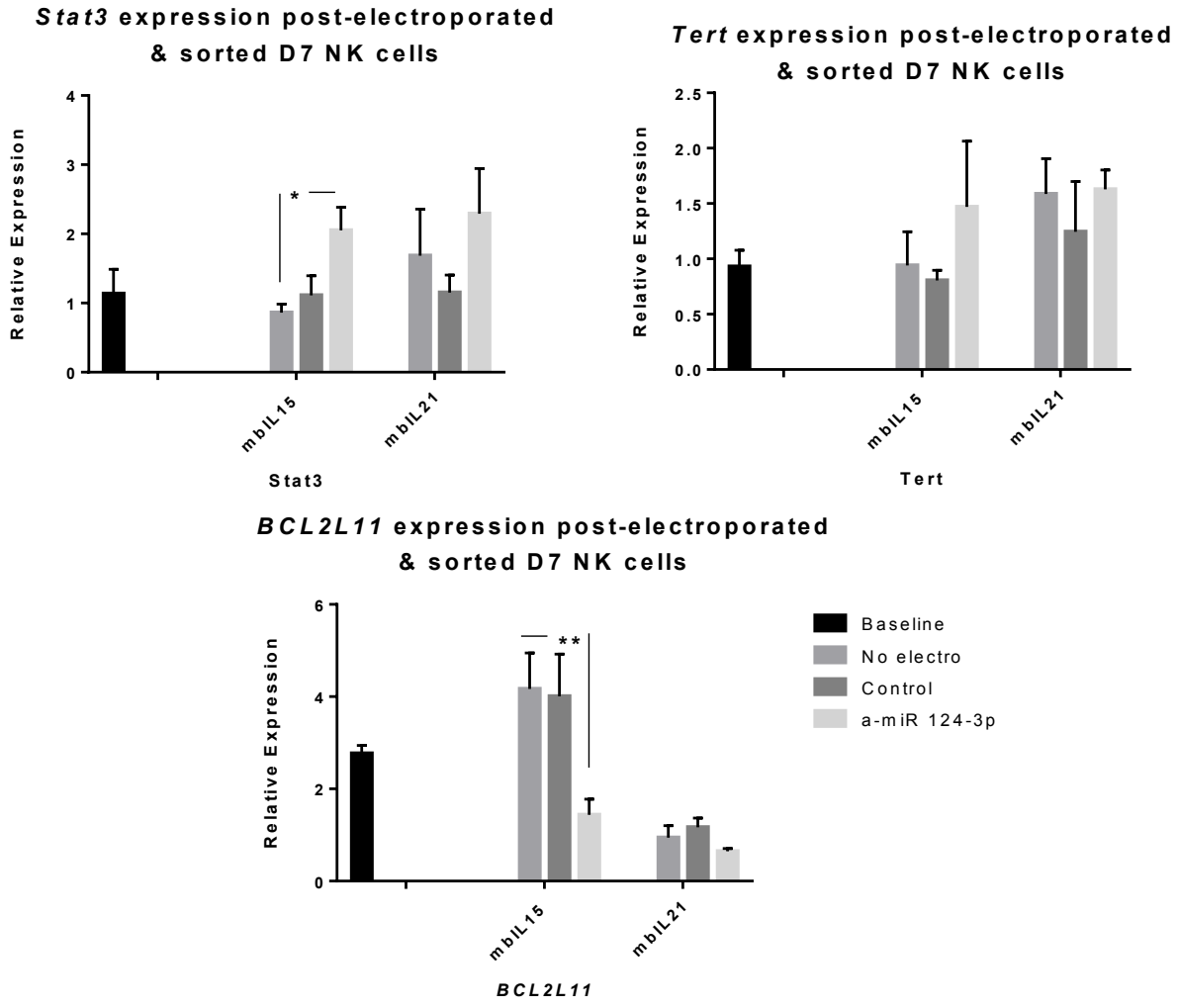


Figure 27: Expansion of NK cells with mbI15 after knockdown of miR124-3p increases STAT3 and TERT, and decreases BCL2L11 in NK cells. Similar to the experiment above, NK cells were electroporated with negative control or anti-miR 124-3p, and NK cells were expanded for 1 week with either mbIL15 or mbIL21. The cells were sorted for CD56 and NKp46 after 7 days, and the effect of IL-15 signaling on NK cells was measured by the expression of STAT3, TERT and BCL2L11 by qRTPCR. As shown, STAT3 and TERT expression in NK cells increased, and BCL2L11 expression decreased despite mbIL15 expansion, corroborating the increased proliferation and decreased apoptosis observed.

The expression level of hTERT is critical and rate limiting to telomerase activity and telomere length, and its expression is primarily by transcriptional control (193-195). Thus, in order to establish the molecular mechanism of telomerase regulation, it is critical to understand the transcriptional and post transcriptional regulation of hTERT. So far, although telomere length and TERT expression changes have been observed in NK cells, it has not been empirically shown to be due to any contributing factor. This is the first report showing the regulation of TERT in expanded NK cells through miRNA.

Finally, we validated our gene expression findings after miR 124-3p knockdown, by measuring the protein expression in NK cells. Similar to gene expression studies, we electroporated the NK cells with negative control or anti-miR 124-3p, and assessed the expression of Stat3, Tert, Bim and phospho-Stat (Y705 and Y727) by western blot. Corresponding with he gene expression data, we observed that Stat3, Tert and phospho-Stats expression increased whilst Bim expression decreased (Figure 28).

Taken together, we have shown through this study that downregulation of miR 124-3p in IL-21 mediated expansion of NK cells is responsible for the higher expansion observed. Knockdown of miR 124-3p and expansion using IL-15 was able to partially restore proliferation, understandably because of the transient nature of knockdown.

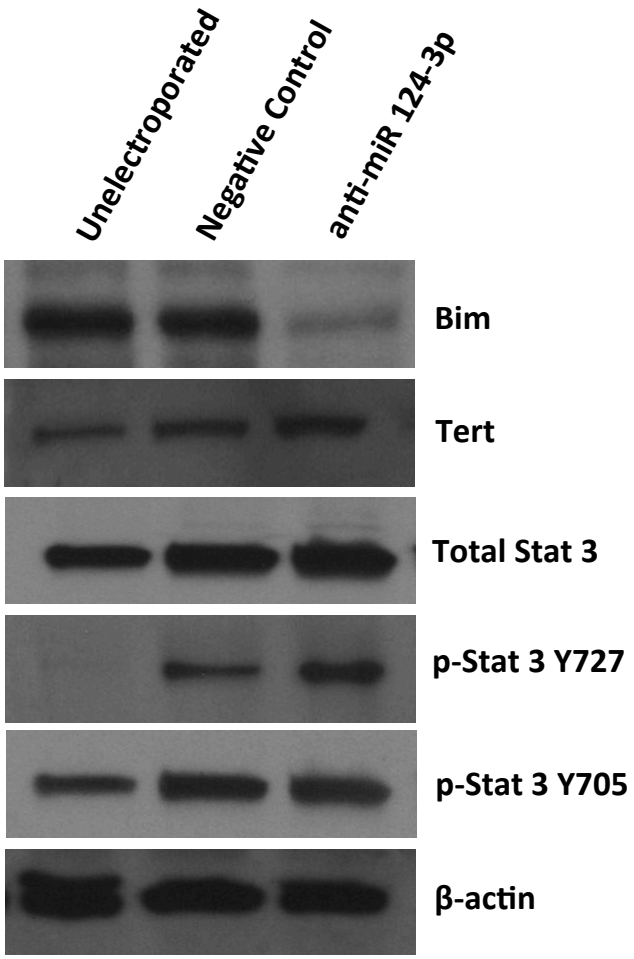


Figure 28: Knockdown of miR 124-3p Increases Stat 3, p-Stat3 and Tert Protein Expression and Decreases Bim Protein Expression. NK cells were electroporated with negative control and anti-miR 124-3p, and protein was collected and estimated after 48 hours. Western blot analysis was performed to probe for Stat3, Tert, p-Stats and Bim; β-actin was used for loading control. As shown, the expression of Stat3, Tert p-Stat3 Y705 and p-Stat3 Y-727 increased, and Bim expression decreased due to miR 124-3p knockdown, corroborating our findings with increased proliferation, and decreased apoptosis in NK cells.

CHAPTER 5: SUMMARY AND CONCLUSION

NK cells are lymphocytes of the innate immune system, and are attractive candidates for adoptive immunotherapy. Owing to the small percentage of NK cells in the peripheral blood, several expansion methods have been developed including K562 based membrane bound IL15 and membrane bound IL21 developed in or lab. K562.mbIL21 far exceeds he expansion potential of K562.mbIL15, whilst maintaining phenotype and cytotoxic functions. Considering the effectiveness of mbIL21-mediated expansion of functionally potent NK cells, it is definitively advantageous to use this system for clinical applications, where permissible.

The sustained proliferative advantage of IL-21 signaling ex vivo arises from the continual activation of Stat3 by IL-21, and promotion of sustained phosphorylation of Stat3. The role of Stat3 in increased proliferation and anti-apoptotic signals in tumor cells are well established. Contrary to IL-21, Stat5 signaling activated by IL-15. Interestingly, Stat5 has also been shown to be activated by IL-21, with more sustained activation by IL-1. This clearly indicates an advantage to the IL-21 mediated pro survival signal in NK cells. We and others have previously established the role of STAT 3 and STAT 5 in mediating signaling through IL-21 and IL-15, respectively, and we have shown that it is possible that STAT 3 signaling and possible consequent maintenance of telomere length, promotes robustness of expansion in the mbIL21 platform. Through this project we sought to specifically address the observed differences and the possible cellular processes that drive these differences due to IL-15 and IL-21 signaling. We proposed that the differences between mbIL15 and mbIL21 could be due to post-transcriptional regulation, particularly through miRNA. To address this, we performed

global expression analyses of mRNA, protein and miRNA, and identified key differential factors through stringent statistical analyses.

In addition, Stat3 is involved in driving almost all of the pathways that control NK cytolytic activity as well regulatory interactions between NK cells and other components of the immune system (196). Stat3 regulates all aspects of NK cell biology, including NK development, activation, target cell killing, and fine-tuning of the innate and adaptive immune responses, as well as critically regulating the pathways and soluble factors activated in tumor-associated NK cells, cancer cells, and regulatory myeloid cells, thus collectively determining the outcome of cancer immunity. Additionally IL-21 signaling and its resultant Stat3 activation has been shown to promote secretion of cytokine and pro-inflammatory mediators including IL-6, IFN-γ, IL-17, and CCL5, granzyme B and perforin, thus reiterating the importance of IL-21 signaling in NK cells. Further, it has also been shown that miRs such as miR 146-3p, miR 27a, miR 155 which are differentially expressed between mbIL15 and mbIL21 are involved in the modulation of 1 or more of these cytokines.

This data provided us insight into the overall expression pattern, as well as the potential for evaluating miRNA in the regulation of other critical aspects of NK cells such as cytotoxicity, cytokine production and ADCC. However, since the focus of this project is to specifically understand the influence of miRNA in mediating expansion of NK cells through IL-15 and IL-21, we intend to use this database and the findings in future studies. We discovered BCL2L11, Bim and miR 124-3p to be the most differentially expressed, with highest expression in IL-15 expanded NK cells.

Based on literature evidence on miR 124-3p's role as a tumor suppressor, and its role in causing tumorigenesis through increased proliferation and decreased apoptosis, particularly via activating STAT3 in various tumor types, and based on the expression pattern in mbIL15 and mbIL21 NK cells, we hypothesized that knockdown of miR 124-3p followed by expansion with mbIL15 would increase Stat3, Tert and reduce Bim, and thus restore the proliferative potential of NK cells expanded on IL-15. To accomplish this, we performed transient knockdown of miR 124-3p through electroporation, and demonstrated that indeed knockdown of miR 124-3p followed by stimulation with mbIL15 increased the overall expansion of NK cells. Further we showed that decreasing miR 124-3p modulated the expression levels of key functional genes and proteins involved in mediating expansion of NK cells.

Through this study, we have demonstrated that upregulation of miR 124-3p due to IL-15 signaling in mbIL15 expansion, drives increased apoptosis and decreased proliferation of NK cells mediated by increased BCL2L11 and Bim, and decreased Stat3, Tert and p-Stat3, respectively, thereby significantly diminishing the overall expansion potential of these cells (Figure 29).

Furthermore, we have demonstrated through this study that downregulation of miR 124-3p due to IL-21 signaling in mbIL21 expanded NK cells plays a major role in upregulating Stat3 and Tert, and downregulating Bim, thereby driving the prolific expansion of NK cells through increased proliferation and decreased apoptosis (Figure 30).

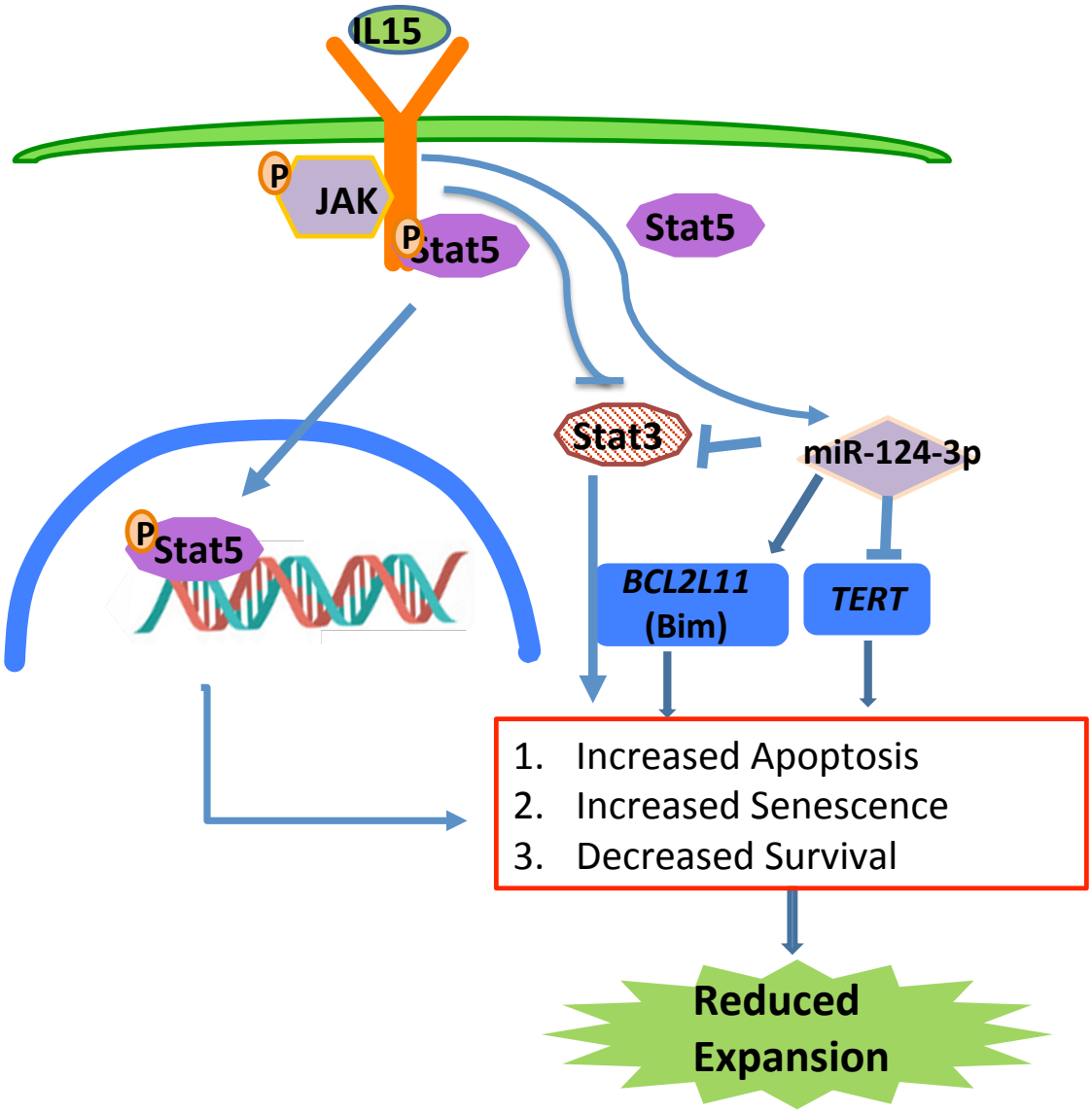


Figure 29: Schematic of modified IL-15 mediated signaling in NK cells. Through this study, we have discovered the role of miR 124-3p in modulating the expression of Stat3, Bim and Tert, thereby driving the differential expansion potential in NK cells. As shown, IL-15 signaling upregulates miR 124-3p, which in turn activates Bim, and suppresses Tert and Stat3, leading to an overall reduced expansion of NK cells due to continual IL-15 signaling.

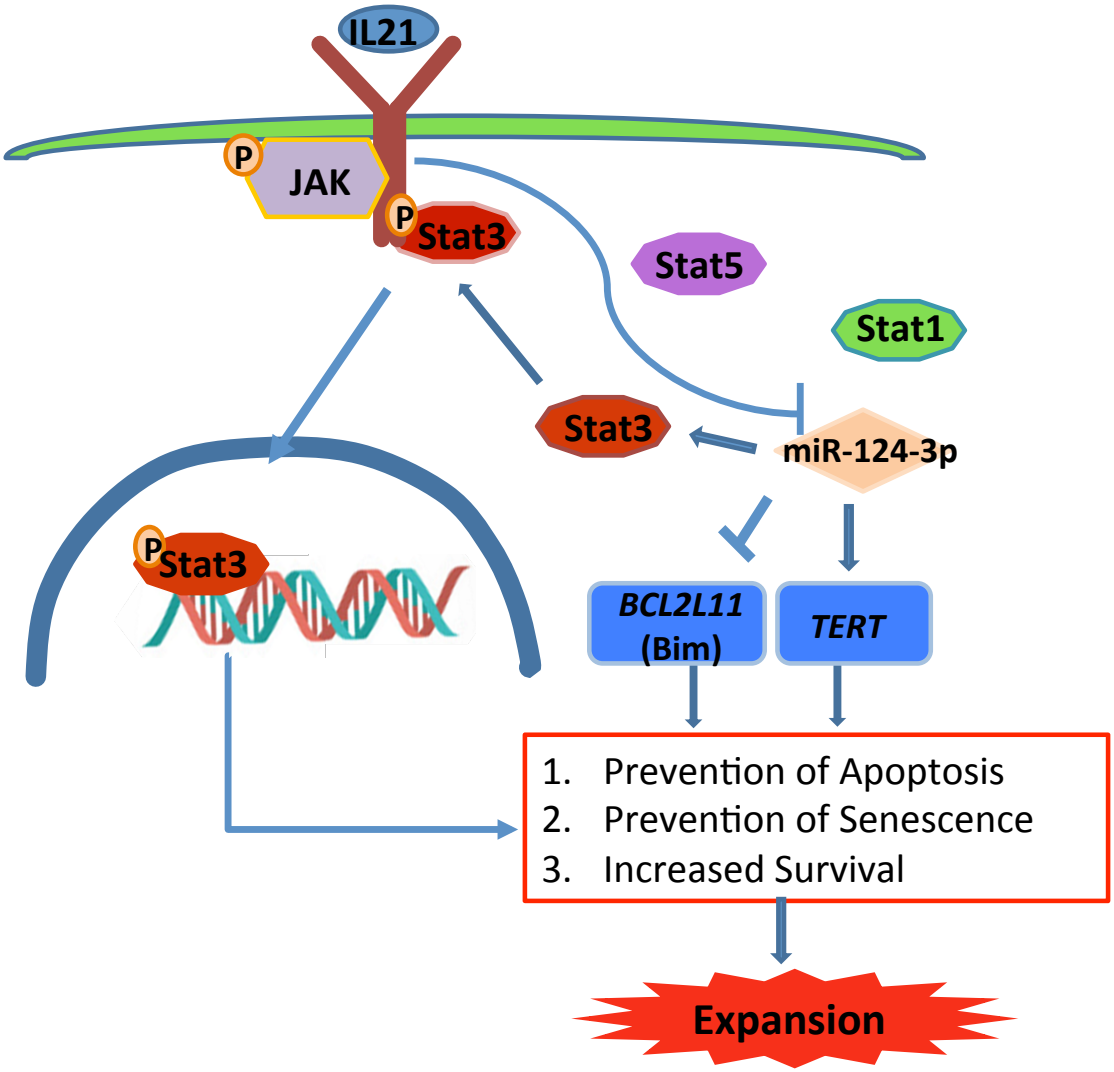


Figure 30: Schematic of modified IL-21 mediated signaling in NK cells. The role IL-21 signaling in downregulating miR 124-3p, and consequent effect on the overall expansion is shown here. IL-21 signaling decreases miR 124-3p, which regulates increased expression of Stat3, Bim and Tert, thereby drives the differential expansion potential in NK cells. As shown, IL-21 signaling downregulates miR 124-3p, which in turn suppresses Bim, and activates Tert and Stat3, leading to an overall prolific increase in expansion of NK cells due to continual IL-21 signaling.

Finally, through this study, we have demonstrated for the first time that miR 124-3p knockdown in mbIL15 NK cells has a significant effect in increasing expression of STAT3 and TERT, and consequent proliferation of NK cells. We also showed that knockdown of miR 124-3p in mbIL15 NK cells had a significant effect in decreasing expression of BCL2L11 (Bim), and consequent apoptosis. We demonstrated that knockdown of miR 124-3p in mbIL15 NK cells had a significant effect in improving overall expansion of NK cells (Figure 31).

In conclusion, we have demonstrated the role of miR 124-3p in NK cells for the first time in this study. We have also demonstrated the key role that miR 124-3p plays in modulating expressions of Tert and Bim in NK cells for the first time through this study. Additionally, we have demonstrated the role of miR 124-3p in modulating Stat3 expression in NK cells for the first time. We have established that overexpression of miR 124-3p and consequent overexpression of Bim (BCL2L11) is responsible for reduced expansion potential in mbIL15 NK cells. We also demonstrated that expansion of NK cells on mbIL21 overcomes the intrinsic expression of miR 124-3p and consequent Bim (BCL2L11), thus able to support robust expansion. We have finally demonstrated that by knockdown of miR 124-3p and consequent downregulation of Bim (BCL2L11), as well as increased Stat3 and Tert, we are able to support better expansion of mbIL15 NK cells.

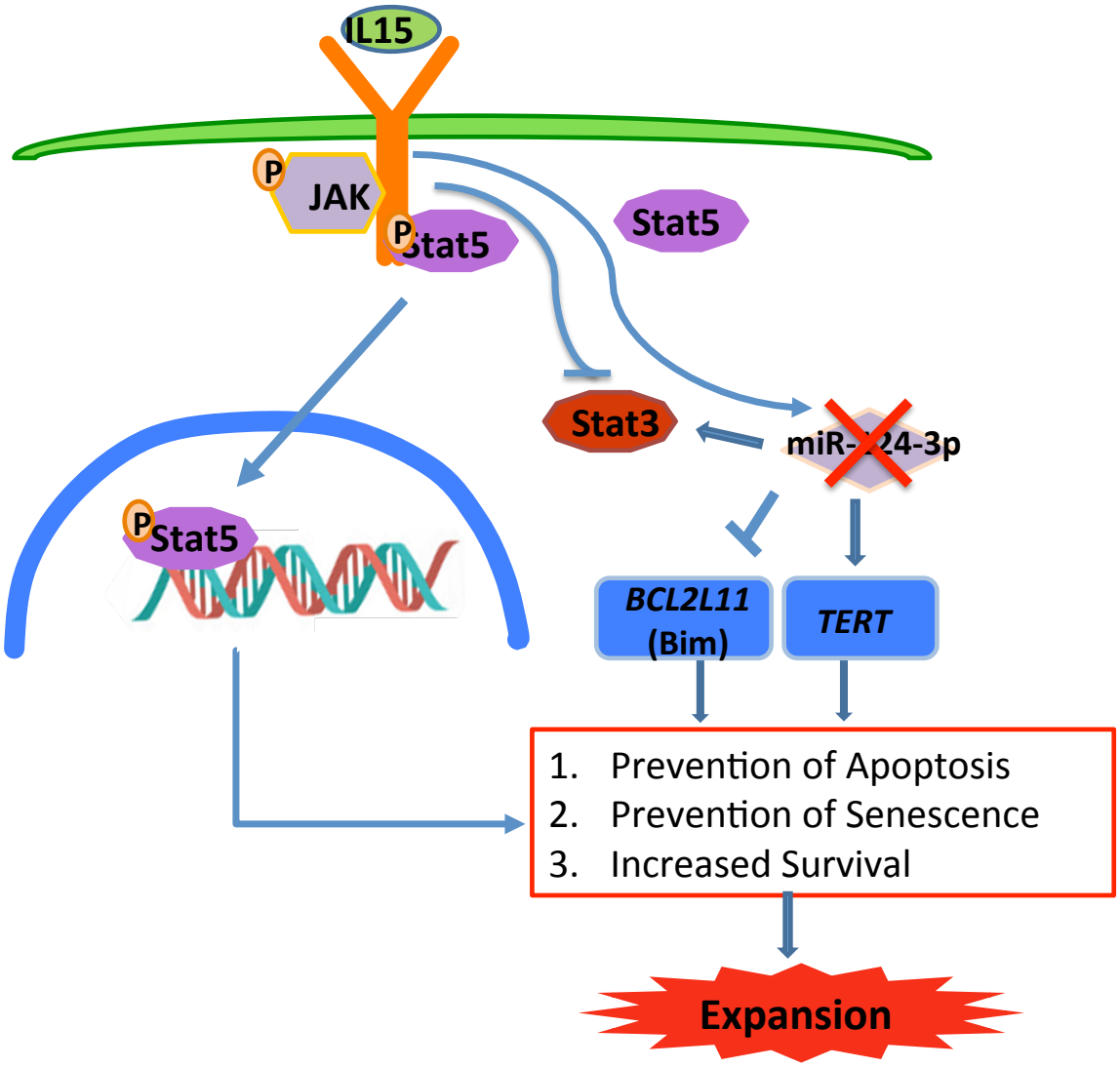


Figure 31: Schematic of the effect of miR 124-3p knockdown on improving the overall expansion potential of mbIL15 NK cells. As shown, knockdown of miR 124-3p, restores the proliferative potential and decreases apoptosis due to continual IL-15 signaling, thereby improving the expansion of NK cells. Furthermore, the impact of miR 124-3p in modulating proliferation and apoptosis through Stat3, Tert and Bim is demonstrated here.

Impact of Work

In this study, we performed a global analysis of miRNA, gene and protein expressions between freshly isolated NK cells and NK cells expanded on membrane bound IL15 and membrane bound IL21. We identified miRNA that differentially regulate several aspects of NK cells including cellular proliferation, apoptosis, cytolytic activity, cytokine secretion and senescence. Additionally, we identified genes and proteins that are similarly regulating these aspects, and correlated them to the miRNA expression. While study and comparison of any of these aspects of NK cell biology and function would be relevant and of significance, we specifically focused on the most differential quality between NK cells expanded on these 2 platforms. The overall impact of this study is twofold: Firstly we have demonstrated a mechanistic insight into the robustness of expansion of NK cells with K562.mbIL21, and the role of IL-21 mediated-signaling in downregulating miR 124-3p, thereby, promoting proliferation and inhibition of apoptosis. Secondly, we have shown that knockdown of miR 124-3p overcomes the intrinsic drawback of apoptosis and reduced proliferation seen with expansion of NK cells with K562.mbIL15, thereby promoting better expansion with mbIL15.

CHAPTER 6: FUTURE DIRECTIONS

In this study, we have defined the role of miR 124-3p in causing the differential expansion of NK cells with membrane bound IL-15 and IL-21. We specifically focused on the expansion potential of ex vivo expanded NK cells for this project. We have shown that Stat3, Tert and Bim expressions are regulated through miR 124-3p, and that miR 124-3p expression is mediated by IL-15 and IL-21 signaling differentially. The regulation of Stat3 by miR 124-3p has been shown previously to be a direct effect in tumor types. However, the regulation of Tert and Bim could be either direct or through indirect mechanisms (through intermediary molecules). The list of players in these pathways with regard to IL-15, IL-21 signaling and miR 124-3p needs to be further elucidated.

In this study, we employed a transient knockdown method for studying the effect of miR 124-3p in the proliferative and apoptotic outcome in NK cells. It would be interesting to see if using lentiviral transduction or CRISPR mediated knockout of miR 124-3p may have a different outcome. Additionally, it would be insightful to see if any of the other differentially expressed miRNAs act either synergistically or opposite to that of miR 124-3p in his model.

Based on the differential gene and protein expression in NK cells, we addressed the role of miR 124-3p in modulating Bim expression, in addition to Stat 3 and Tert. However, it has ben shown that downregulation of miR 124-3p is involved in promoting cell cycle progression through upregulation of CDK6 and Rb genes, as well as preventing checkpoint inhibition from G0-G1 phase; additionally, downregulation of miR 124-3p has also been

shown to enhance the activation of anti-apoptotic proteins. (192, 197). Finding the correlation between miR 124-3p and these pathways would add more strength to the proliferation aspect of this study.

Further, it would be interesting to understand how miR 124-3p-mediated Stat3 regulation affects NK cells functioning. IL-15 signaling is mediated through Stat-1, Stat-3 or Stat-5, although there is a preferential signaling through Stat-5. It would be interesting to see if increased availability of Stat 3 due to miR 124-3p suppression during the initial phase of NK cell expansion could skew the signaling preference for IL-15 from Stat 5 to Stat 3. If this were to happen, NK cell expansion with IL-15 would continue more robustly without the impeding apoptotic and anti-proliferative signals.

Finally, with regard to expansion, it would also be interesting to see if miR 124-3p or any other miRNA influenced by IL-15 or IL-21 signaling play a role in regulating homeostasis in NK cells (ex: miR 142). If such a miRNA could be identified, it would be beneficial for modulating NK cell homeostasis not only in this model, but also would be beneficial in studying NK cell-related autoimmunity.

Although we explored the role of miRNA, gene, and protein expression related to expansion, our global expression analyses of fresh and expanded NK cells provides a dataset with which to address the role of miRNA in regulating various other cellular functions in NK cells including differential cytokine secretion due to IL-15 and IL-21 signaling (project design and execution under consideration), cytotoxicity, and differential ADCC. Additionally, comparison of fresh peripheral blood NK cells' miRNA and gene expression could be

compared with either mbIL15 or mbIL21 expanded NK cells to address the underlying mechanisms of cellular activation and increased function in NK cells.

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VITA

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